Proapoptotic Triterpene Electrophiles (Avicins) Form Channels in Membranes: Cholesterol Dependence

Xiao Xian Li,* Bridgette Davis,* Valsala Haridas,† Jordan U. Gutterman,† and Marco Colombini*
*Department of Biology, University of Maryland, College Park, Maryland; and †Department of Molecular Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

ABSTRACT Avicins, a family of triterpenoid saponins from *Acacia victoriae*, can regulate the innate stress response in human cells. Their ability to induce apoptosis in transformed cells makes them potential anticancer agents. We report that avicins can form channels in membranes. The conductance reached a steady state after each addition, indicating a dynamic equilibrium between avicin in solution and in the membrane. The high power dependence (up to 10) of the membrane conductance on the avicin concentration indicates the formation of multimeric channels, consistent with the estimated pore radius of 1.1 nm. This radius is too small to allow protein flux across the mitochondrial outer membrane, a process known to initiate apoptosis. Channel formation is lost when avicin's amphipathic side chain is removed, implicating this as the channel-forming region. A small difference in this side chain results in strong cholesterol dependence of channel formation in avicin G that is not found in avicin D. In neutral membranes, avicin channels are nonselective, but negatively-charged lipids confer cation selectivity (5:1, K^+:Cl^-), indicating that phospholipids form part of the permeation pathway. Avicin channels in the mitochondrial outer membrane may favor apoptosis by altering the potential across this membrane and the intermembrane space pH.

INTRODUCTION

Avicins (structure in Fig. 1) are a recently discovered family of triterpenoid electrophiles that regulate the innate stress response in human cells (Joshi et al., 2002; Jayatilake et al., 2003). For example, the avicins have been shown to induce apoptosis by direct perturbation of mitochondria (Haridas et al., 2001b; Mujoo et al., 2001), activating the intrinsic caspase pathway. Recent results (V. Haridas, X. X. Li, M. Higuchi, M. Colombini, and J. U. Gutterman, unpublished data) demonstrate that the avicins favor the closure of the outer mitochondrial membrane channel, VDAC, resulting in suppression of nucleotide exchange and decrease in oxygen consumption. Postmitochondrial events during apoptosis include suppression of HSP70 and HSP90 by activation of the ubiquitin/proteasome pathway (A. Gaikwad, A. Poblenz, V. Haridas, and J. U. Gutterman, unpublished data). Avicins' proapoptotic effects are accompanied by a cytoprotective effect on healthy cells. The NRF-2 transcriptional factor is activated by a redox mechanism and thus stimulates expression of a gene battery enhancing cell detoxification and antioxidant effects (Haridas et al., 2004). As part of the regulation of stress, the avicins also suppress multiple proinflammatory components of the innate immune system, including the transcriptional factor NF-κB (Haridas et al., 2001a), the Pi3K/AKT signaling pathway, as well as heat shock proteins. In mouse skin models, the avicins have been shown to suppress the initiation and promotion phase of chemical carcinogenesis as well as UVB damage with resultant suppression of oxidative DNA and lipid damage (Hanausek et al., 2001). Thus, the avicins represent a new class of metabolites that regulate cellular redox balance through a coordinated system response to cellular stress.

Here we present results that characterize the ability of avicin to form channels in membranes. This somewhat surprising result introduces another dimension that needs to be considered when interpreting changes in cellular function. This article is not intended to definitively relate this channel-forming property to the induction of apoptosis, but a speculation on a possible mechanism is offered.

MATERIALS AND METHODS

Diphytanoylphosphatidylcholine (DPhyPC) and polar extract of soybean phospholipids (asolectin) were purchased from Avanti Polar Lipids (Alabaster, AL). Avicins were purified as previously described (Haridas et al., 2001b) and stored refrigerated in aliquots appropriate for one day of use. Dry aliquots were generated by dissolving avicin in water and lyophilizing. Working solutions (1 mg/ml) to be used on the same day were generated by freshly dissolving the avicin in the appropriate medium, generally the same medium used on the *cis* side of the planar membrane experiment (see below). The solution was kept on ice until needed. All other reagents were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma Chemical (St. Louis, MO).

Reconstitution experiments in planar phospholipid membranes

Planar phospholipid membranes were formed by the monolayer method of Montal and Mueller (1972) as revised by Colombini (1987). A phospholipid membrane was formed across a 0.1-mm-diameter hole in a Saran partition using monolayers composed of phospholipids (either DPhyPC or asolectin, or a combination of these, plus or minus cholesterol as indicated). The membrane separated two 5-mL aqueous compartments named *cis* and *trans* containing 0.10 M KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.0 (unless otherwise indicated). The voltage was applied to the *cis* side, and the *trans*

Submitted July 13, 2004, and accepted for publication January 5, 2005.

Address reprint requests to Marco Colombini, Tel.: 301-405-6925; Fax: 301-314-9358; E-mail: colombini@umd.edu.

0006-3495/05/04/2577/08 $2.00

doi: 10.1529/biophysj.104.049403

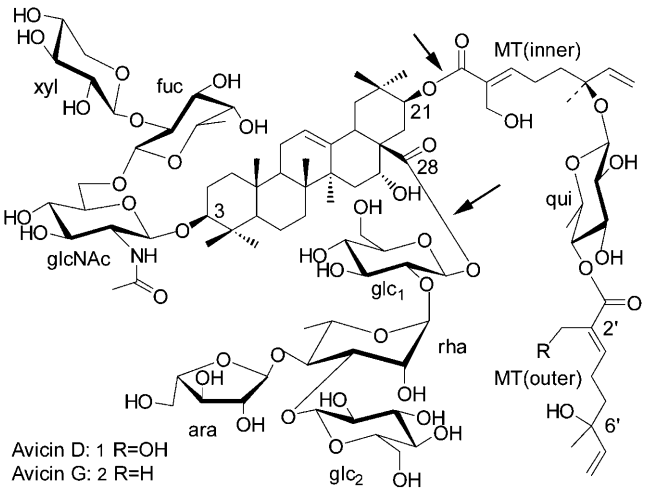

FIGURE 1 Structures of avicins used in this work. The arrows indicated the hydrolysis sites of the truncated avicin. (For details in these structures, see Jayatilake et al., 2003.)

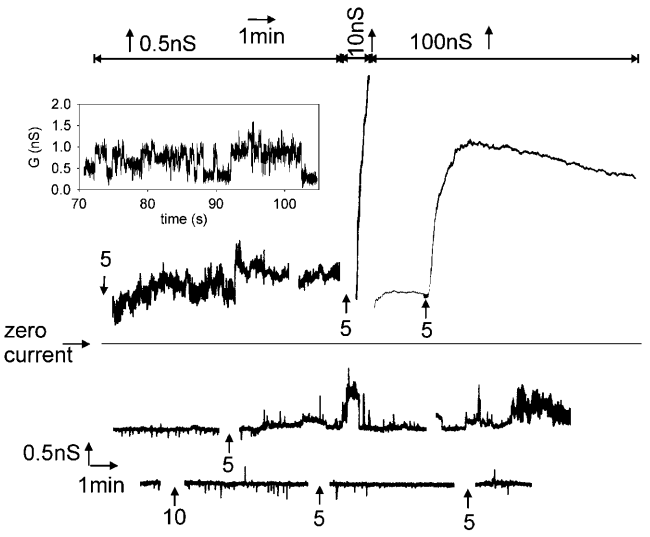

FIGURE 2 Conductance increase in a planar phospholipid membrane after the sequential addition of avicin G to the aqueous medium on the *cis* side of the membrane. The membrane was formed from monolayers consisting of asolectin/cholesterol (10:1 w/w). The record begins at the lower left and then continues from left to right and bottom to top. Avicin G was added at the time points indicated by the short arrows with the numbers indicating the number of micrograms added to the 5 ml of solution in the *cis* compartment. The first 3 additions (10, 5, and 5 μg) resulted in no increase in conductance (*bottom trace*). After the fourth addition (5 μg) the conductance increased and returned to baseline (*second trace* from the *bottom*). Further additions caused sustained increases in conductance (*third trace*). Note the frequent scale changes: the current scale is indicated by the longer vertical arrow and was changed twice in the upper trace. The inset shows an expanded record of single-channel activity filtered at 50 Hz.

side was maintained at virtual ground by an amplifier in the inverted mode (52 K; Analog Devices, Norwood, MA). The current was recorded both on a chart recorder and with the Axotape recording system (Vers. 2, Axon Instruments, Foster City, CA). The current was filtered at a frequency of 20 Hz using a Butterworth filter. Calomel electrodes with built-in saturated KCl bridges were used to interface with the aqueous solutions.

Erythrocyte lysis assay

Erythrocytes were obtained from decapitated rats (Sprague-Dawley males) with added EGTA to prevent clotting. The cells were washed by successive centrifugations from 150 mM NaCl, 4 mM EGTA, and 5 mM HEPES, pH 7.4 and resuspended in the same buffer. The erythrocyte suspension was diluted 1:20 with an equiosmolal solution of the test non-electrolyte. The final erythrocyte concentration was such that 400 μL of it would result in an absorbance of 0.15 at 540 nm after reaction with the Drabkins reagent (Sigma Technical Bulletin No. 525, Sigma Chemical). To 500 μL of the erythrocyte suspension, 5 μL of avicin (1 mg/mL) was added and the cells sedimented (2 min at 12,000 *g*) after a prescribed period of time (generally 5 min). A 400-μL aliquot of supernatant was mixed with 400 μL of Drabkins reagent and the absorbance at 540 nm measured after 5 min.

RESULTS

Channel formation by avicin G

The addition of avicin G (freshly solubilized in an aqueous solution) to an aqueous compartment bathing a phospholipid membrane resulted in increases in the conductance of the membrane. The highly cooperative nature of the formation of the permeability pathway is evident in Fig. 2. Significant conductance was observed only after four additions of a total of 25 μg of avicin G to 5 ml of solution on the *cis* side of the membrane. At this level of avicin, the conductance often returned to baseline. After the next 5-μg addition (*left end* of *upper trace*), the conductance increased to a noisy steady-state level. A further addition (*middle* of *upper trace*) and the conductance increased so rapidly that the scale was reduced by a factor of 20 and then another factor of 10. Once the conductance had stabilized, a further 5-μg addition resulted in the conductance reaching a new higher steady state. The inset shows that the conductance increase is the result of discrete conductance steps characteristic of channels. Thus the conductance was the result of the formation of aqueous channels rather than a carrier mechanism. The conductance leveled off after each addition of avicin G, although sometimes the steady-state conductance was preceded by an overshoot. Presumably the overshoot is due to a bolus of added avicin hitting the membrane. These observations indicate the presence of a dynamic equilibrium between the channels in the membrane and the monomers in aqueous solution.

Although the samples used were highly purified, the avicins were obtained from a natural source and thus there is always the concern that a minor contaminant might be responsible for the conductance. However, stable conductances could easily be achieved well into the microSiemen range (comparable to the electrode resistance), thus greatly reducing the likelihood that a contaminant could be responsible (the contaminant would have to be extremely potent).

Channel formation may be the result of the presence of the acyclic monoterpine side chain. The addition of a modified

version of avicin G in which the side chain was cleaved (Fig. 1) resulted in no detectable formation of channels in the membrane up to a final concentration in the aqueous phase of 25 μg/ml (five times the amount needed to see a conductance with avicin G). Beyond that, an increase in membrane conductance was observed but it had very different properties. A smooth increase in conductance characteristic of a carrier was observed (data not shown). Thus, rather than the formation of channel-like pathways, the truncated molecule may have resulted in the transport of ions, one or a few ions at a time.

The increase in conductance with addition of normal avicin G was obviously nonlinear (Fig. 3). Further additions increased the conductance in a logarithmic fashion, indicating that multiple avicin G molecules were required to generate the permeability pathway. From the slopes of the logarithmic plots that ranged from 4 to 12, the entire process is highly cooperative.

The channels show no significant rectification (Fig. 4). The Ohmic behavior is unlikely to arise from equal numbers of channels oriented in opposite directions because the avicin was added only to one side (*cis*) and, in view of the extensive glycosylation of the molecule, avicin is unlikely to be able to cross the membrane easily. If any did cross, they would likely move into the aqueous medium on the *trans* side because this would essentially act as an infinite sink.

Selectivity of avicin G channels

Experiments were performed in the presence of a salt gradient to determine the ion selectivity of the avicin G channels. When neutral phospholipids were used, the reversal potential was almost zero, indicating no selectivity between K^+ and Cl^- ions (Fig. 5). However, when a membrane containing negatively-charged lipids was used, the channels showed substantial cation selectivity. Using the Goldman-Hodgkin-Katz equation, the permeability ratio was 5:1. The dependence of the selectivity on negatively-charged lipids indicates that these contribute to the channel structure.

The selectivity for cations did not change appreciably as the conductance of the membrane increased (Fig. 5). This indicates that the conductance increase was the result of the formation of more channels rather that the formation of larger channels.

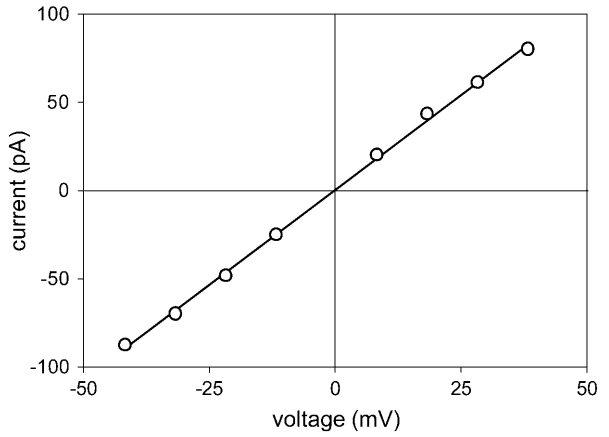

FIGURE 4 Avicin G channels show an Ohmic relationship between current and voltage. The total conductance of avicin G channels was 2.1 nS. Avicin G was added to the *cis* side only. The membranes were formed from a lipid composition of 41.5% DPhyPC, 41.5% asolectin, and 17% cholesterol (by weight).

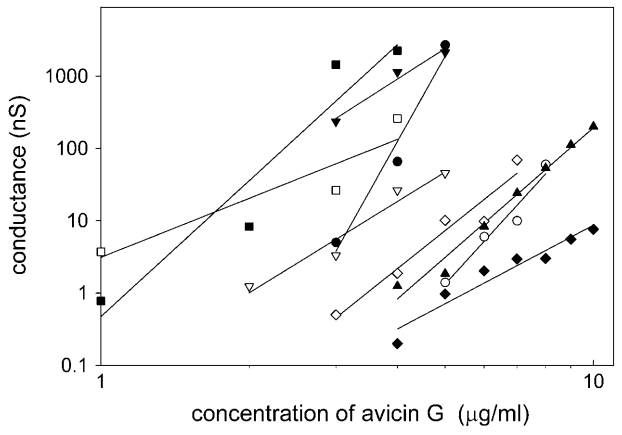

FIGURE 3 Conductance generated in planar phospholipid membranes by the addition of avicin G to the aqueous phase on the *cis* compartment. All membranes were made from monolayers containing 83% asolectin and 17% cholesterol by weight. The medium was 0.10 M KCl, 1 mM $MgCl_2$, and 5 mM PIPES, pH 7.0. Each line and its associated symbols indicates a separate experiment. Sequential additions of avicin G were made after the conductance had reached a steady value. The avicin concentration is the total concentration in the aqueous compartment assuming no partitioning into the membrane.

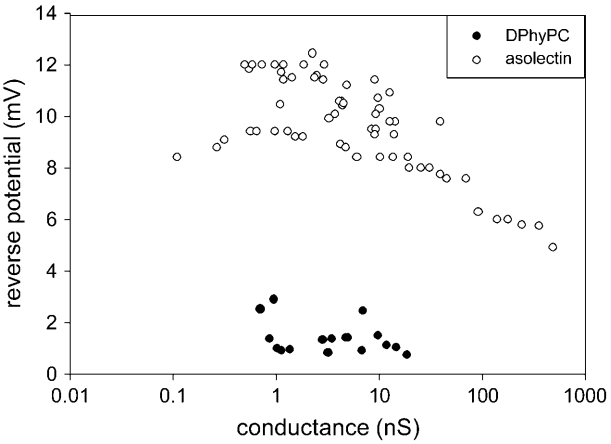

FIGURE 5 Selectivity of avicin G channels. The membrane, either DPhyPC or asolectin with 17% cholesterol (by weight), as indicated, was made separating 0.20 M and 0.10 M KCl. The reversal potential is reported as voltage in the low salt side minus that in the high salt side. The asolectin data represents four independent experiments and the DPyPC is from two independent experiments.

Size of the aqueous pathway formed by avicin G channels

The size of these channels was determined by adding avicin G to erythrocytes. The cells were suspended in iso-osmotic solutions consisting of non-electrolytes of different molecular size. The percent lysis was measured by sedimenting the intact cells and measuring hemoglobin released from lysed cells. If the avicin channels are permeable to the test non-electrolyte, the resulting osmotic imbalance will lyse the erythrocytes. Fig. 6 shows that the cutoff was between polyethylene glycol (PEG) 1000 and PEG 1500. In the inset, one can see that, with time, more lysis was observed with PEG 1000, but that lysis in the presence of PEG 1500 leveled off, indicating a slow permeation by PEG 1000. Doubling the dose of avicin did not change the size cutoff. The Stokes-Einstein radii and the radii of gyration of these molecules are 1.0 and 1.2 nm for PEG 1000 and PEG 1500, respectively. The clear cutoff shows that avicin G forms channels of a discrete size.

Concentration dependence

The conductance of the membrane depends strongly on the concentration of avicin added to the aqueous phase (Fig. 7), indicating that multiple avicins must cooperate to generate a conductive path. This is not surprising in view of the large pathway formed and the small size of the avicin molecule. The situation is more complex because the concentration dependence varies with the lipid composition. The conductance induced by avicin G showed a much stronger concentration dependence in membranes made from mixed soybean lipids (asolectin) with a net negative charge as compared to neutral lipids (DPhyPC). More accurate estimates of the concentration dependence were obtained by averaging the slopes from the log/log plots. As illustrated in Fig. 8, in the absence of cholesterol there is a fivefold decrease

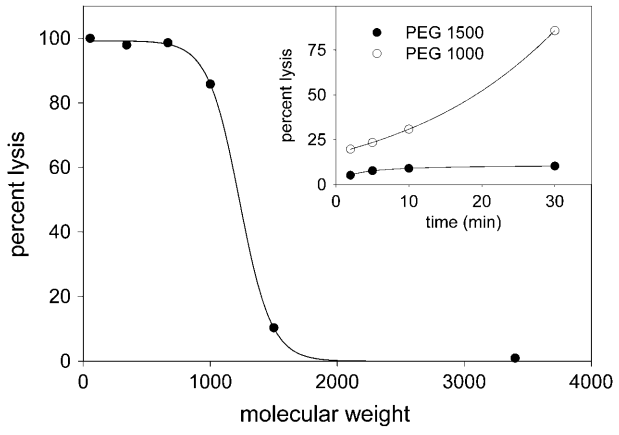

FIGURE 6 Sizing of avicin G channels. Erythrocyte lysis was used to determine the pore size of the avicin G channels formed in their plasma membranes. See Materials and Methods.

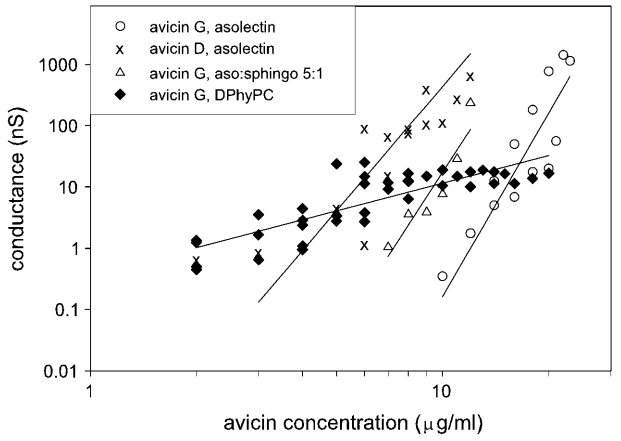

FIGURE 7 Avicin-induced conductance in phospholipid membranes without cholesterol. The indicated lipids were used to make the membrane. (*Aso*:*sphingo* means asolectin/sphingomyelin.) The slopes from the linear regression are: 9.9, 8.8, 1.5, and 6.7 for avicin G in asolectin, asolectin/sphingomyelin, DPyPC, and avicin D in asolectin, respectively.

in the power dependence of conductance on concentration of avicin G in the two lipids. In the presence of cholesterol, these differences become statistically insignificant.

Cholesterol dependence

The propensity for channel formation by avicin G depends strongly on the amount of cholesterol present in the

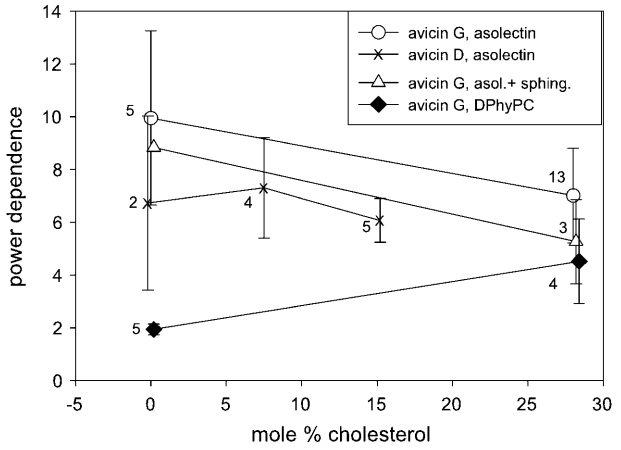

FIGURE 8 The power dependence of the conductance on the avicin added. The indicated lipids were used to make the membrane. The *asol+sphing* was 5:1 asolectin/sphingomyelin. *Mol % cholesterol* is the percent of the moles of lipid in the membrane-forming solution that were cholesterol. The error bars are mean ± SE of the number of independent experiments indicated next to the symbols. The power dependence of the conductance of avicin G in DPhyPC membranes without cholesterol was much less than in all other conditions (*t*-test versus asolectin no cholesterol, $P < 0.04$; and versus asolectin 28 mol % cholesterol, $P < 0.02$). In the presence of cholesterol, the difference in the power dependence between DPhyPC and asolectin membranes disappeared ($P > 0.25$).

membrane (Fig. 9). The presence of cholesterol shifts the dose-response curve threefold along the concentration axis but this results in an increase in conductance of 10,000-fold in any given concentration. Yet, the dependence of the conductance on the avicin G added is essentially the same with and without cholesterol, indicating the formation of similar structures.

Avicin D is almost identical to avicin G (Fig. 1), but shows no dependence on cholesterol (Fig. 9). This is remarkable in view of the small structural difference between the two molecules.

To quantitatively estimate the relative potency of the avicins in different lipid environments, we calculated the dose required to obtain a conductance of 10 nS. This was calculated for each separate experiment and averaged (Fig. 10). Note that only avicin G in asolectin showed a very strong cholesterol dependence on its ability to form channels. Other trends were not statistically significant. These differences in potency are also evident in Fig. 7. Note that if a higher conductance was used as a benchmark, the preference for channel formation in asolectin over DPhyPC would be much more evident.

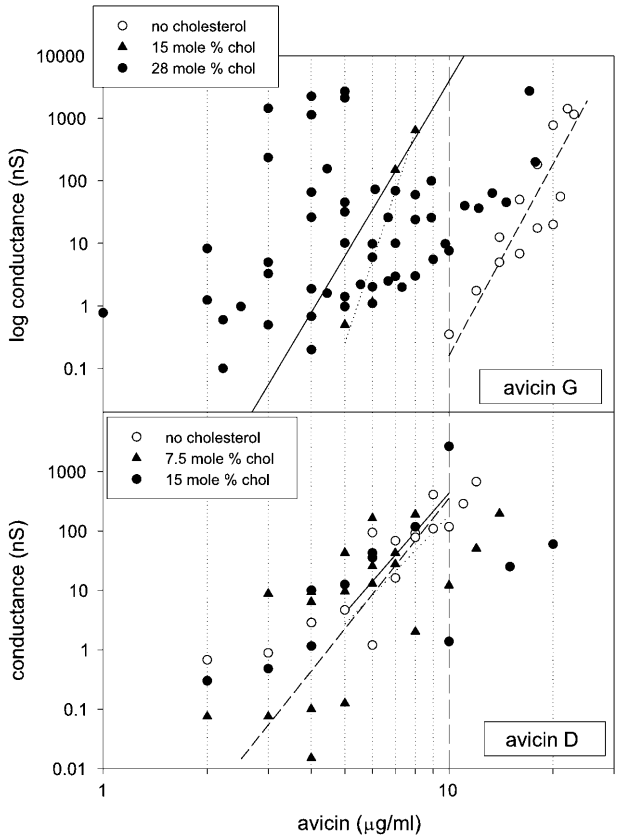

FIGURE 9 Cholesterol influences the dependence of the conductance on the amount of added avicin G but not avicin D. Other than cholesterol, the lipid used to make the membrane was asolectin. The lines fitted to the data were generated by obtaining the average slope and average intercept from individual experiments (each weighted by the number of data points in that experiment). For avicin G, the fitted lines for 0%, 15%, and 28% cholesterol have slopes and doses that yield 10 nS (in μg/ml), equal to 9.4 $\pm$ 0.7 (3) and 15 $\pm$ 0.6 (3); 17 and 6.2; and 7 $\pm$ 1.8 (13) and 4.5 $\pm$ 0.8 (13), respectively. For avicin D, the fitted lines for 0%, 7.5%, and 15% cholesterol have slopes and doses that yield 10 nS (in μg/ml) equal to 7 $\pm$ 3 (2) and 6 $\pm$ 1 (2); 7 $\pm$ 2 (5) and 6 $\pm$ 1 (5); and 6.1 $\pm$ 0.8 (4) and 6 $\pm$ 2 (4), respectively. Results expressed as mean $\pm$ SE (number of independent experiments).

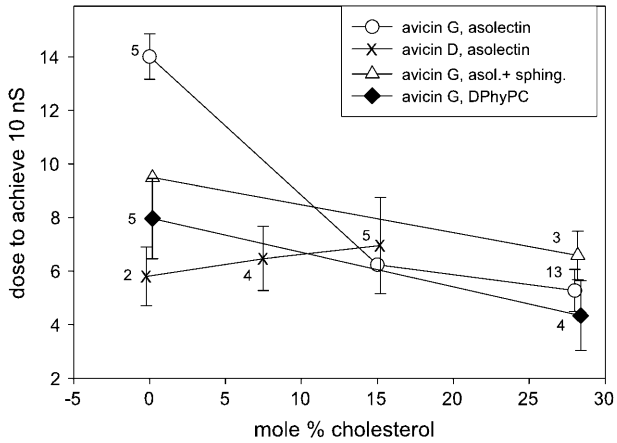

FIGURE 10 Influence of cholesterol on the dose (in μg/ml) needed to achieve a conductance of 10 nS in a planar membrane of the specified composition. These doses were obtained from conductance/concentration curves. When not specified, the phospholipid used was asolectin. The sphingomyelin-containing membrane was composed of asolectin/sphingomyelin at a 5:1 ratio. This was held constant as the cholesterol level was increased. The error bars are standard errors of the mean of the number of independent experiments indicated next to the symbols. In asolectin without cholesterol, a much higher dose was required to achieve the same level of conductance. This was highly significantly different versus asolectin with 28 mol % cholesterol, $P < 0.000006$; DPhyPC, $P < 0.006$, with no cholesterol; $P < 0.0009$, 28 mol % cholesterol; avicin D, $P < 0.02$, with no cholesterol; $P < 0.002$, 15 mol % cholesterol; and $P < 0.007$, 28 mol % cholesterol.

DISCUSSION

Structurally, avicins are part of a greater family of molecules called triterpenoid saponins. Some of these are toxic primarily through their detergent-like activity, whereas others alter membrane structure resulting in specific changes in cell function, such as activation of secretion (Melzig et al., 2001). The avicins seem to be in the latter category. A key to understanding their mode of action may lie in their ability to form channels in specific membranes in cells. In this work we have sought to characterize the channels formed by avicins and to begin exploring the conditions that favor or disfavor channel formation.

Avicins are water-soluble, but are sufficiently amphipathic to partition into membranes. Unlike detergents, avicins increase the membrane permeability to ions without destabilizing the membranes to any significant extent. The observed conductance depends on the amount of avicin added to the aqueous phase resulting in clear, stable conductance levels. This behavior is characteristic of amphipathic

structures that self-assemble in the membrane to form well-defined structural entities.

Erythrocytes provide a convenient system to measure the pore size of the avicin channels by using non-electrolytes. Both avicin G and avicin D self-assemble into channels with an estimated pore size of 1.1 nm in radius. Slow erythrocyte lysis in PEG 1000 shows that this molecule's hydrodynamic radius closely matches the narrowest part of the aqueous pore. This was seen with both avicins, indicating a similar channel-forming structure. The fact that the selectivity of avicin channels in membranes containing negatively-charged lipids remained rather constant as the conductance was changed over three orders of magnitude, shows that avicin channels exist at an optimum size, and that increases in conductance reflect increases in the number of channels rather than in the size of channels.

The major difference between avicin G and D is the sensitivity to the presence of cholesterol. The presence of a single additional hydroxyl group in the extended side chain of avicin D replaces the need for cholesterol to increase the propensity for channel formation. Since, without cholesterol, avicin D forms channels of almost identical properties to those formed by avicin G with cholesterol, this indicates that, for avicin G, cholesterol is more of a regulator than an essential part of the channel-forming pathway. Saponins, in general, have been shown to bind to cholesterol (Shany et al., 1974; Osbourn et al, 1996), and thus avicin D may still bind cholesterol—but if it does, the cholesterol is not influencing the propensity to form channels.

The dependence of avicin G channels on cholesterol may explain the ability of avicin G to induce apoptosis selectively in tumor cells. Low levels induce apoptosis in Jurkat cells (Haridas et al., 2001b) but are cytoprotective in normal cells (Haridas et al., 2001a, 2004; Hanausek et al., 2001). The reason for this differential effect may lie in the higher cholesterol content in the mitochondrial outer membranes of tumor cells (Galeotti et al., 1986). The cholesterol content in tumor cell mitochondria is approximately four times that of normal cell mitochondria (Parlo and Coleman, 1984).

Clearly, other differences in the composition of said membranes may be responsible for the observed specificity. In addition, lack of knowledge about the activity of cholesterol in biological membranes (separate from the cholesterol composition) hampers one's ability to predict which membranes might be more sensitive to permeabilization by avicin G. Another factor may be the presence of substances, in solution or on the membrane, that sequester avicin monomers preventing the free avicin from achieving the critical concentration required for channel formation. Nevertheless, the correlation between avicin's channel-forming ability and its ability to induce apoptosis exists, and is strengthened by the observation that truncated avicin G does not induce apoptosis in Jurkat cells (Jayatilake et al., 2003).

Although avicin G can induce cytochrome-c release from mitochondria (Haridas et al., 2001b), our measurements demonstrate that its pore size is too small to allow cytochrome c release. A variety of both anti- and pro-apoptotic regulators form channels so avicin may act in a similar way. Alternatively, avicin may interact with other proteins and lipids that have been implicated as forming the pathways from the selective release of proteins from mitochondria in the early phases of apoptosis: e.g., BAX and ceramide (Schlesinger et al., 1997; Siskind et al., 2002).

From an examination of the structure of the avicins one might not suspect that these would form channels in membranes. The highly glycosylated triterpene nucleus should ensure that it is anchored to the aqueous phase on the surface of the membrane. Thus, the amphipathic extended side chain is an attractive candidate for the transmembrane portion of the avicin that might form the walls of the channel. Indeed, triterpenoid saponins with an acyl side chain have been shown to increase membrane permeability of endothelial cells, whereas those lacking the side chain did not (Melzig et al., 2001). This finding is consistent with our results in that the removal of the acyl side chain of avicin G results in the loss of all channel-like behavior. Thus, we propose that the avicin channels are essentially barrels composed of extended side chains that span the membrane (Fig. 11). The number of these chains would be given by the power dependence of the conductance on the avicin concentration (6–10 depending on the conditions used). On one end of the channel (on the side of avicin addition) the triterpene portions of the molecules must pack together to form some sort of ring structure. This structure may or may not form the end of the permeability pathway. At the other end, headgroups of phospholipids should form an annulus through which permeating ions must pass to cross the membrane. This annulus could be responsible for the selectivity of avicin in negatively-charged asolectin membranes. A related triterpenoid saponin,

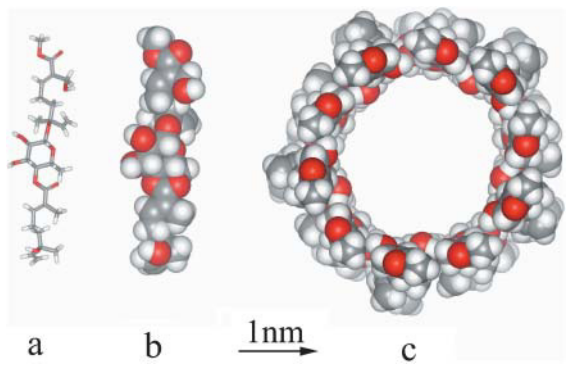

FIGURE 11 Model of the channel formed by avicin G. The illustration shows only the side chain of avicin G. The extended conformation (*a* and *b*) can be assembled so as to optimize packing and hydrogen-bond formation. The illustrated 10-strand pore (*c*) is in conformity with the power dependence of the conductance on the amount of avicin added. Interestingly, the assembled structure forms a lumen whose diameter is consistent with the size estimates determined experimentally.

avenacin A-1, was shown to permeabilize planar phospholipid membranes (Armah et al., 1999), and this conductance was interpreted as being due to channel formation.

It is not easy to reconcile the observations reported here with the actions of avicin in intact cells. The ability of avicins to permeabilize erythrocyte membranes raises the question of why avicin does not kill cells by necrosis. Perhaps the membrane trafficking system internalizes avicin from the plasma membrane and it finds its way to the mitochondrial outer membrane. This has actually been shown to happen in HeLa cells exposed to meningococci (*Neisseria meningitides*), where a channel called Por-B moves from the bacterial outer membrane to the plasma membrane and then travels to the mitochondrial outer membrane and inhibits apoptosis (Massari et al., 2003). Note that here also the cells do not undergo necrosis. The details of how this process works are under investigation.

Avicins induce apoptosis and this can be partly explained by a direct effect of avicin favoring VDAC closure (V. Haridas, X. X. Li, M. Higuchi, M. Colombini, and J. U. Gutterman, unpublished data), because closure of VDAC channels has been shown to be an early and reversible event leading to mitochondria-mediated apoptosis (Vander Heiden et al., 2001). It is not obvious, however, how channel formation by avicins can lead to the induction of apoptosis. The following is a speculative proposal that could guide future research. Consider that many Bcl-2 family proteins, both pro- and anti-apoptotic, form channels (Minn et al., 1997; Schlesinger et al., 1997), and the role of channel formation is unclear save for the obvious formation of a pathway for protein flux. The anti-apoptotic protein Bcl-x_L forms cation selective channels (Minn et al., 1997), whereas the pro-apoptotic proteins form channels that are either nonselective or weakly anion selective (Schlesinger et al., 1997). Respectable theoretical calculations show that metabolic flux across the mitochondrial outer membrane coupled with differential permeability to the permeating ions (small ions and metabolic ions) can lead to a substantial potential across the mitochondrial outer membrane (Lemeshko and Lemeshko, 2000; Lemeshko, 2002). The potential would act in at least two ways: 1), favor the closure of the voltage-gated VDAC channels located in this membrane; and 2), result in a pH in the intermembrane space that differs from that of the cytosol (protons will equilibrate with the potential). These effects could lead to the initiation of apoptosis by either VDAC closure and/or a pH-dependent activation or inhibition of enzymes leading to apoptosis. An example of the latter could be ceramide-induced apoptosis where the steady-state level of mitochondrial ceramide would be controlled by the relative activities of enzymes that generate and consume ceramide (Siskind et al., 2002). Thus, by changing the selectivity of the mitochondrial outer membrane, avicin could alter the potential across this membrane, leading to conditions favoring apoptosis. Obviously, the complexity of this proposal, and particularly the influence of membrane lipids, allow plenty of room for tumor cells to be hypersensitive to avicin.

REFERENCES

Armah, C. N., A. R. Mackie, C. Roy, K. Price, A. E. Osbourn, P. Bowyer, and S. Ladha. 1999. The membrane-permeabilizing effect of avenacin A-1 involves the reorganization of bilayer cholesterol. *Biophys. J.* 76: 281–290.

Colombini, M. 1987. Characterization of channels isolated from plant mitochondria. *Methods Enzymol.* 148:465–475.

Galeotti, T., S. Borrello, G. Minotti, and L. Masotti. 1986. Membrane alterations in cancer cells: the role of oxy radicals. *Ann. N. Y. Acad. Sci.* 488:468–480.

Hanausek, M., P. Ganesh, Z. Walaszek, C. J. Arntzen, T. J. Slaga, and J. U. Gutterman. 2001. Avicins, a family of triterpenoid saponins from *Acacia victoriae* (Bentham), suppress H-*ras* mutations and aneuploidy in a murine skin carcinogenesis model. *Proc. Natl. Acad. Sci. USA.* 98: 11551–11556.

Haridas, V., C. J. Arntzen, and J. U. Gutterman. 2001a. Avicins, a family of triterpenoid saponins from *Acacia victoriae* (Bentham), inhibit activation of nuclear factor-κB by inhibiting both its nuclear localization and ability to bind DNA. *Proc. Natl. Acad. Sci. USA.* 98:11557–11562.

Haridas, V., M. Higuchi, G. S. Jayatilake, D. Bailey, K. Mujoo, M. E. Blake, C. J. Arntzen, and J. U. Gutterman. 2001b. Avicins: triterpenoid saponins from *Acacia victoriae* (Bentham) induce apoptosis by mitochondrial perturbation. *Proc. Natl. Acad. Sci. USA.* 98:5821–5826.

Haridas, V., M. Hanausek, G. Nishimura, H. Soehnge, W. Zbigniew, M. Narog, E. Spears, R. Zoltaszek, and J. U. Gutterman. 2004. Triterpenoid electrophiles (avicins) activate innate stress response by redox regulation of a gene battery. *J. Clin. Invest.* 113:65–73.

Jayatilake, G. S., D. R. Freeberg, Z. Liu, S. L. Richheimer, M. E. Blake, D. T. Bailey, V. Haridas, and J. U. Gutterman. 2003. Isolation and structures of avicins D and G: potent tumor-inhibiting saponins derived from *Acacia victoriae*. *J. Nat. Prod.* 66:779–783.

Joshi, L., J. M. Van Eck, K. Mayo, R. DiSilvestro, M. E. Blake, T. Ganapathi, V. Haridas, J. U. Gutterman, and C. J. Arntzen. 2002. Metabolomics of plant saponins: bioprospecting triterpene glycoside diversity with respect to mammalian cell targets. *OMICS.* 61:235–246.

Lemeshko, S. V., and V. V. Lemeshko. 2000. Metabolically derived potential on the outer membrane of mitochondria: a computational model. *Biophys. J.* 79:2785–2800.

Lemeshko, V. V. 2002. Model of the outer membrane potential generation by the inner membrane of mitochondria. *Biophys. J.* 82:684–692.

Massari, P., C. A. King, A. Y. Ho, and L. M. Wetzler. 2003. Neisserial PorB is translocated to the mitochondria of HeLa cells infected with *Neisseria meningitidis* and protects cells from apoptosis. *Cell. Microbiol.* 5:99–109.

Melzig, M. F., G. Bader, and R. Loose. 2001. Investigations of the mechanism of membrane activity of selected triterpenoid saponins. *Plant Med.* 67:43–48.

Minn, A. J., P. Vélez, S. L. Schendel, H. Liang, S. W. Muchmore, S. W. Fesik, M. Fill, and C. B. Thompson. 1997. Bcl-x_L forms an ion channel in synthetic lipid membranes. *Nature.* 385:353–357.

Montal, M., and P. Mueller. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA.* 69:3561–3566.

Mujoo, K., V. Haridas, J. J. Hoffmann, G. A. Wächter, L. K. Hutter, Y. Lu, M. E. Blake, G. S. Jayatilake, D. Bailey, G. B. Mills, and J. U. Gutterman. 2001. Triterpenoid saponins from *Acacia victoriae* (Bentham) decrease tumor cell proliferation and induce apoptosis. *Cancer Res.* 61:5486–5490.

Osbourn, A. E., P. Bowyer, and M. J. Daniels. 1996. Saponin detoxification by plant pathogenic fungi. *Adv. Exp. Med. Biol.* 404:547–555.

Parlo, R. A., and P. S. Coleman. 1984. Enhanced rate of citrate export from cholesterol-rich hepatoma mitochondria. The truncated Krebs cycle and other metabolic ramifications of mitochondrial membrane cholesterol. *J. Biol. Chem.* 259:9997–10003.

Schlesinger, P. H., A. Gross, X. M. Yin, K. Yamamoto, M. Saito, G. Waksman, and S. J. Korsmeyer. 1997. Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic Bcl-2. *Proc. Natl. Acad. Sci. USA.* 94:11357–11362.

Shany, S., A. W. Bernheimer, P. S. Grushoff, and K. S. Kim. 1974. Evidence for membrane cholesterol as the common binding site for cereolysin, streptolysin O and saponin. *Mol. Cell. Biochem.* 3: 179–186.

Siskind, L. J., R. N. Kolesnick, and M. Colombini. 2002. Ceramide channels increase the permeability of the mitochondrial outer membrane to small proteins. *J. Biol. Chem.* 277:26796–26803.

Vander Heiden, M. G., X. X. Li, E. Gottleib, R. B. Hill, C. B. Thompson, and M. Colombini. 2001. Bcl-x_L promotes the open configuration of VDAC and metabolite passage through the mitochondrial outer membrane. *J. Biol. Chem.* 276:19414–19419.