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Actin Modulates the Gating of *Neurospora crassa* VDAC

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Abstract. VDAC forms the major pathway for metabolites across the mitochondrial outer membrane. The regulation of the gating of VDAC channels is an effective way to control the flow of metabolites into and out of mitochondria. Here we present evidence that actin can modulate the gating process of *Neurospora crassa* VDAC reconstituted into membranes made with phosphatidylcholine. An actin concentration as low as 50 nM caused the VDAC-mediated membrane conductance to drop by as much as 85% at elevated membrane potentials. Actin's effect could be quickly reversed by adding pronase to digest the protein. α-Actin, from mammalian muscle, has a stronger effect than β- and γ-actin from human platelets. The monomeric form of actin, G-actin, is effective. Stabilization of the fibrous form, F-actin, with the mushroom toxin, phalloidin, blocks the effect of actin on VDAC, indicating that F-actin might be ineffective. Cytochalasin B did not interfere with the ability of actin to favor VDAC closure. DNase-I did effectively block actin's effect on VDAC, and VDAC decreased actin's inhibitory effect on DNase-I activity, indicating that *N. crassa* VDAC competes with DNase-I for the same binding site on actin. The actin-VDAC interaction might be a mechanism by which actin regulates energy metabolism.

Key words: Mitochondria — Outer membrane — Metabolism — G-actin — VDAC — Channel

Introduction

Mitochondria are not distributed randomly within a cell. Their size, shape, and location vary dramatically with cell type and with the metabolic or functional state of the cell. Mitochondria have also been shown to be moved from one portion of the cell to another both by microtubule and microfilament-based motility. The classical work in this area is the motion of mitochondria down axons on microtubules. More recently mitochondria have also been shown to move along actin filaments both in axons (Morris & Hollenbeck, 1995) and in yeast cells during cell division (Simon, Swayne & Pon, 1995).

The site of attachment of mitochondria to the cellular cytoskeleton is only partly understood. There is evidence in the literature for binding of microtubule-associated proteins (MAPS) to the mitochondrial outer membrane channel called VDAC (or mitochondrial porin) (Linden & Karlsson, 1996). Evidence for a specific mitochondrial site for binding to the actin cytoskeleton of yeast has been reported (Boldogh et al., 1998). Here we present evidence that the major pathway across the outer mitochondrial membrane, called VDAC, binds directly to G-actin.

VDAC is a 30 kDa protein located in the mitochondrial outer membrane. VDAC forms the pathway by which metabolites cross this membrane. A growing body of evidence indicates that the gating of VDAC channels can control the flux of important metabolites such as ATP and this could result in regulation of mitochondrial functions. For instance, recent reports by Rostovtseva and Colombini (Rostovtseva & Colombini, 1996, 1997) show that the closure of this channel totally shuts down the movement of ATP while allowing small ions to permeate. The deletion of yeast VDAC proteins greatly decreases the permeability of the outer membrane in isolated yeast mitochondria (Lee et al., 1998).

A number of cytosolic and intermembrane space proteins have been shown to bind to VDAC. Some isoforms of hexokinase and glycerol kinase bind to certain VDAC isoforms (Lindén, Gellerfors & Nelson, 1982; Fiek et al., 1982). Creatine kinase, located in the intermembrane space, has been shown to bind to mammalian

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VDAC (Brdiczka, Kaldis & Wallimann, 1994). Another intermembrane space protein, referred to as the VDAC modulator, binds to and controls the properties of VDAC (Liu & Colombini, 1991, 1992). Here, we report that actin can also modulate the gating of *Neurospora crassa* VDAC channels.

Materials and Methods

Materials

Neurospora crassa mitochondria were isolated as previously described by Mannella (1982) and *N. crassa* VDAC protein was purified (Blachly-Dyson et al., 1990) and dissolved in 15% DMSO, 2.5% Triton X100, 50 mM KCl, 10 mM Tris, 1 mM EDTA, pH 7.0. Rat liver VDAC protein was purified as described in Colombini (1983).

Beef liver mitochondria were isolated as previously described by Liu et al. (1994) using the method of Rickwood et al. (1987). Yeast cells expressing different VDAC genes were grown and the mitochondria were isolated as described by Lee et al. (1998). Mitochondria were hypotonically shocked by resuspension in 1 mM KCl, 1 mM HEPES, pH 7.0, and mitochondrial membranes were obtained by sedimentation at 18,000 RPM (SS-34 rotor) for 20 min.

G-actin from rabbit muscle was purchased from Worthington Biochemical. G-actin from porcine heart muscle was purchased from Sigma Chemical. G-actin from human platelets (a mixture of 85% β-actin and 15% γ-actin) was purchased from Cytoskeleton. F-actin from rabbit muscle was purified as described by Pardee and Spudich (1982) with additional purification by cycles of depolymerization and repolymerization.

Phalloidin, DNase-I and cytochalasin B were purchased from Sigma Chemical. In the bilayer study, phalloidin and DNase-I were dissolved, shortly before use, in the same buffered 1 M KCl medium used for membrane formation (*see below*). Cytochalasin B was dissolved in DMSO shortly before use and the final DMSO concentration in the chamber was less than 1% (v/v). All the other chemicals were purchased from Sigma Chemical or Fisher Scientific and were reagent grade.

Reconstitution Into Planar Phospholipid Membranes

The phospholipid membranes were made by the monolayer method of Montal and Mueller (1972) as modified by Schein, Colombini and Finkelstein (1976) and Colombini (1987). Briefly, a Saran partition with a 0.1-to-0.15 mm diameter hole divided a Teflon chamber into two compartments, named *cis* and *trans*. The partition was coated with 5% (w/v) petrolatum in petroleum ether solution and allowed to dry for 10–15 min. Aqueous solutions (1 M KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.2) were then layered in the bottom of each compartment of the Teflon chamber, and 20–30 μl of a 1% (w/v) diphytanoyl phosphatidylcholine, 0.2% cholesterol solution in hexane was layered on top. Five-to-ten minutes were allowed for hexane evaporation, leaving a phospholipid monolayer on the surface of the water solution in each compartment. The two monolayers were raised to form a phospholipid bilayer across the hole in the partition. Each compartment contained 4-to-4.4 ml of solution.

Once the phospholipid membrane was formed, a 0.5–5 μl aliquot of VDAC solution was added to the *cis* side of the chamber while stirring and the stirring continued for about 25 sec. VDAC inserted spontaneously and was studied under voltage-clamp conditions.

The voltage in the *trans* compartment was maintained at virtual ground by an amplifier and the desired voltage was applied to the *cis* side (Colombini, 1987). Calomel electrodes with saturated KCl bridges were used to interface with the solutions. The membrane potential was kept at −10 mV when VDAC or other samples were added to the chamber. Typically, a triangular voltage wave of 5.6 mHz was applied to assess the voltage-gating of VDAC channels.

DNase-I Activity Assay

The activity of DNase-I in the presence of actin and mitochondrial membrane containing different VDAC proteins was measured by using the method previously described by Grinstein and Cohen (1983). Calf thymus DNA fibers were cut with scissors, dissolved to 50 μg/ml in 150 mM Tris-HCl, 4 mM $MgSO_4$, 1.8 mM $CaCl_2$, pH 7.5, and stirred overnight in the cold room (4°C). The solution was filtered before use. Porcine heart actin was freshly dissolved to 0.1 mg/ml in the same solution as that for DNA. DNase-I from bovine pancreas was dissolved to about 0.1 mg/ml in 100 mM Tris-HCl, 10 μM phenylmethylsulfonyl fluoride, 0.1 mM $CaCl_2$, pH 7.5 solution.

In the control experiment, 1 ml of DNA solution was added to a cuvette and used as the blank. Three microliter DNase-I solution was added to the DNA solution and mixed. The hydrolysis was followed by measuring the absorbance increase at 260 nm at room temperature.

Inhibition by Actin

Before the addition of DNase-I, 5 or 10 μl porcine heart actin was added to the DNA solution and the mixture was used as the blank. When mitochondrial membranes were present, the membrane was added to the DNA solution first, then the actin was added, the solution was mixed and incubated at room temperature for 10 min before adding DNase-I.

The Assay of Protein Content

The protein content of the F-actin sample from rabbit muscle was measured using the BCA method (Pierce, Rockford, IL) and bovine serum albumin was used as the standard. The protein content of mitochondrial membrane was measured with the same method by first mixing the membrane with Triton X100 (final concentration 1% (w/v)).

Results

Actin Facilitates the Voltage-gating of VDAC Channels

When reconstituted into mixed lipids such as the soybean phospholipids (asolectin), VDAC channels readily gate at both positive and negative potentials. This gating is the result of two gating processes, one operating at each sign of the potential. Channels are more likely to be open at potentials less than 25 mV and more likely closed at higher voltages. In the neutral synthetic lipid, diphytanoylphosphatidylcholine, VDAC channels still gate but require higher voltages. As a result, it is con-

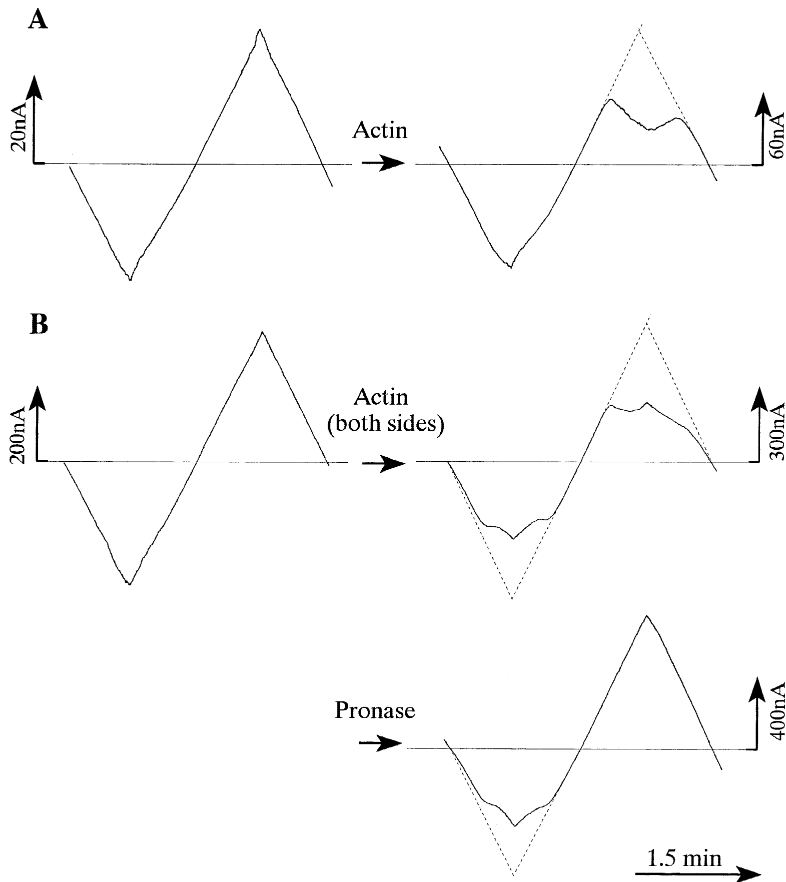

Fig. 1. The effect of F-actin on the gating of *Neurospora crassa* VDAC channels. Membranes were made with the neutral lipid, diphytanoyl phosphatidylcholine. A 63 mV, 5.6 mHz triangular voltage wave was applied to induce VDAC to close. The current records are the solid lines with time proceeding from left to right and the dashed lines are extrapolations of the slope of the traces at low potentials showing the expected result from Ohmic behavior. In panel *A,* VDAC channels hardly closed in the absence of actin. When 24 μg F-actin from rabbit muscle was added to the *trans* compartment, it caused the VDAC channels to close much more easily at positive voltages. The closure of VDAC at negative voltages was much less affected. The data shown were collected 24 min after the addition of actin. In Panel *B,* about 30 μg F-actin from rabbit muscle was added to both sides of membrane. This caused VDAC channels to close at both positive and negative voltages. The segment shown was recorded about 30 min after the addition of actin. The subsequent addition of 1 mg pronase to the *trans* compartment eliminated actin's effect on the gating of VDAC at positive voltages. The segment shown was recorded about 4 min after adding pronase.

venient to use this lipid to clearly demonstrate the effects of modulating reagents on VDAC gating. By using this lipid and applying lower voltages, the records show almost no voltage gating prior to actin addition.

The sample recordings shown in the figures were collected by applying triangular voltage waves to membranes containing many VDAC channels. In Fig. 1*A,* the left record shows essentially an ohmic response of VDAC to the changing voltage. The current rose and fell following the changes in the applied voltage. Virtually no voltage-gating of channels was visible because the applied voltage (63 mV) was not high enough and the frequency of the wave (5.6 mHz) was high giving the channels insufficient time to close. When 24 μg of filamentous actin (F-actin) was added to the *trans* side of the membrane, the VDAC channels closed readily at positive potentials (deviation from linearity) but closure at negative potentials was much less affected. Therefore only 1 of the 2 gating processes was strongly responsive to *trans* actin, the one that responded to positive potentials. The converse experiment achieved similar result, i.e., actin addition to the *cis* side resulted in obvious gating at negative potentials only (*not shown*). Voltage gating at both potentials was evident following addition of actin to both sides (Fig. 1*B*).

The actin protein was responsible for the observed modulation of VDAC voltage dependence. Not only was the F-actin used highly purified, but the observed effect was sensitive to pronase. Actin is very sensitive to pronase (Fisher et al., 1983) and the addition of 1 mg pronase to the *trans* side eliminated the effect on VDAC gating at positive potentials (Fig. 1*B,* bottom). Complete reversal took 4 min. Similarly, pronase added to the *cis* side eliminated the actin effect on gating at negative potentials (*not shown*). Note that pronase has no detectable effect on *N. crassa* VDAC reconstituted into planar membranes (Holden & Colombini, 1988).

The modulation of VDAC's voltage gating was rather specific for alpha actin. Preparations of monomeric actin (G-actin) from rabbit muscle and porcine heart muscle worked just as well as the F-actin preparation from rabbit muscle (Fig. 2*A*). However, non-muscle actin, a combination of beta and gamma actin isolated from human platelets, had a weaker effect (Fig. 2*B*). By contrast, another negatively charged protein, bovine serum albumin, had no effect when as much as 20 milligrams of protein was added (5 mg/ml final). The addition of 50 μg of F-actin to that same membrane resulted in the augmentation of the voltage-dependent closure of VDAC channels (Fig. 2*C*).

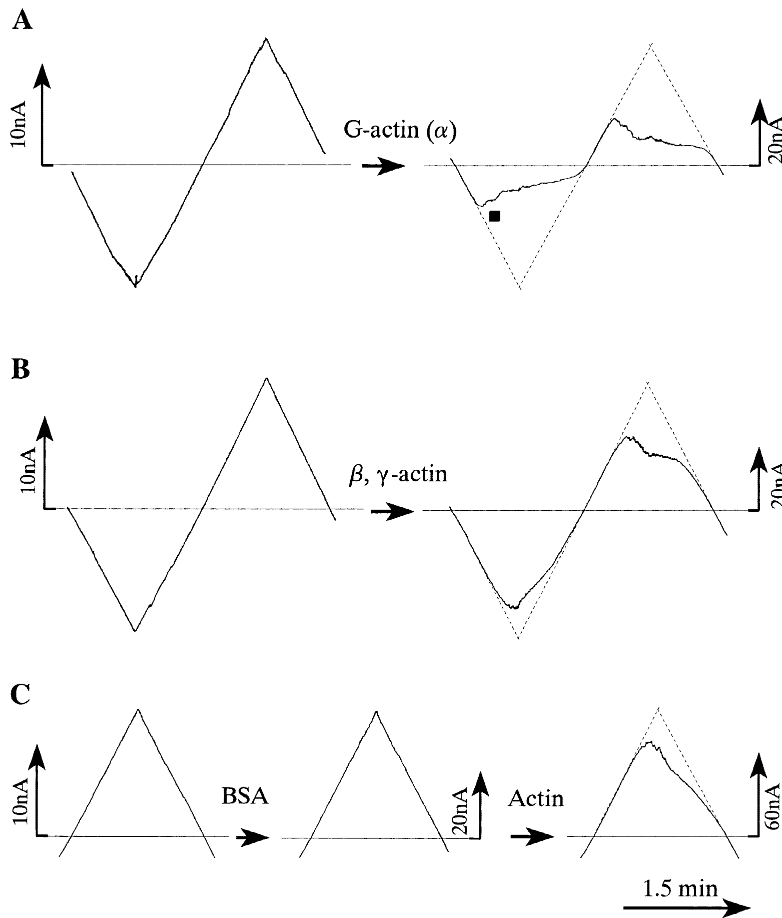

Fig. 2. The effect of different forms of actin on the gating of VDAC channels. The conditions are as indicated in Fig. 1. In panel *A,* 30 μg G-actin from rabbit muscle was added to both sides of the phospholipid membrane containing VDAC channels. This dramatically facilitated the closure of VDAC channels. The record shown was collected 8 min after the addition of actin. In panel *B,* the addition of 50 μg β, γ- actin (a mixture of 85% β-actin and 15% γ-actin) from human platelets to both sides of the membrane caused a smaller effect on VDAC gating. The record shown was collected about 18 min after the addition of actin. In panel *C,* the addition of 20 mg bovine serum albumin (BSA) to the *trans* side of the membrane did not change the gating process of VDAC channels at positive voltage. The record shown was collected 15 min after the addition of BSA. The subsequent addition of 50 μg of F-actin from rabbit muscle caused the expected effect on VDAC gating, although the effect was weaker. The record shown was collected 4.5 min after adding F-actin.

G-actin Is the Form that Modulates VDAC

F-actin is a dynamic molecule and spontaneously depolymerizes in dilute solutions. Since a full response could be obtained with as little as 2 μg/ml of added F-actin (it just took more time, about 7 min instead of less than 4 min), it is likely that the monomer form (G-actin) is responsible for the observed modulation of VDAC channels.

Stabilization of F-actin by preincubation with phalloidin blocked the effect of actin. Since the final bath concentrations of phalloidin used were 5 to 10 μM and thus well above the K_D (40 nM, De La Cruz & Pollard, 1996), it is useful to talk about the molar ratio of phalloidin to actin monomer. At a 20:1 molar ratio, F-actin preincubated with phalloidin had no effect on VDAC (Fig. 3*A*). At a 5:1 molar ratio a weak effect developed with time. In Fig. 3*B,* the left figure resulted from the addition of the phalloidin/actin complex (5:1 molar ratio) to the *cis* side and just F-actin to the *trans* side. The current response at negative potentials is that affected by the actin on the *cis* side. A clear but weaker effect is evident after 20 min. A stronger response with 4 times less actin in the *trans* side is evident at positive potentials. Subsequent phalloidin addition to the *trans* side had no effect (Fig. 3*B,* right). Since phalloidin only binds to F-actin, addition of phalloidin after depolymerization of actin should have no effect on the binding of the monomer to VDAC.

DNase-I is a protein that binds G-actin tightly. An excess of DNase-I (200 μg) was added to the solution bathing a multichannel membrane prior to F-actin addition (Fig. 3*C*). Subsequent addition of F-actin (80 μg) had no effect on the VDAC channels. This can be interpreted as follows. The added F-actin depolymerizes but the resulting actin monomer binds to DNase-I and not to VDAC. Thus there's no effect on the gating of VDAC channels.

Finally, the addition of cytochalasin B, at a 20-fold molar concentration excess, had no effect on actin's ability to augment the voltage sensitivity of VDAC. F-actin preincubated with cytochalasin B still greatly facilitated the gating of VDAC channels (Fig. 3*D*). Cytochalasin B added after the development of the actin effect did not cause any change in actin's effect (*data not shown*).

Actin Modulates VDAC's Gating Process

The records of current as a function of voltage were analyzed by standard methods (Schein et al., 1976; Dor-

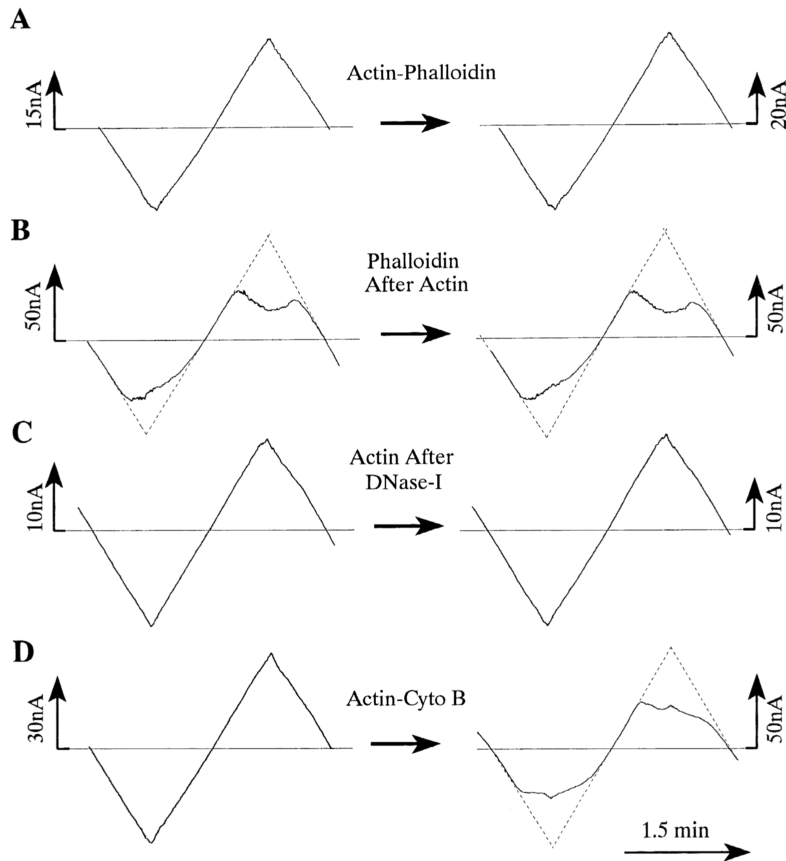

Fig. 3. Pharmacological study of the effect of actin on VDAC channel gating. The conditions are as indicated in Fig. 1. In panel *A,* the addition of 100 μg F-actin from rabbit muscle, preincubated for 10 min with phalloidin (at a molar ratio of 1:20 actin monomer to phalloidin), to both sides of the membrane did not cause any effect on the gating of VDAC channels. The record shown was taken about 15 min after the addition. In panel *B,* the left record shown was collected 21 min after 200 μg F-actin preincubated with phalloidin (at a molar ratio of 1:5) was added to the *cis* side, and 10 min after 50 μg F-actin was added to the *trans* side. The F-actin had a stronger effect on the gating of VDAC (response at positive potentials) than that caused by the F-actin-phalloidin mixture (response at negative potentials). Subsequent addition of 50 μg phalloidin to the *trans* compartment did not change the effect of F-actin. The record shown on the right was taken 7 min after the addition of phalloidin. In panel *C,* there was 200 μg DNase-I in the *cis* compartment and DNase-I had no effect on VDAC gating. Subsequent addition of 80 μg F-actin to the *cis* side did not cause the expected effect, due to the presence of DNase-I. The record shown was taken 15 min after addition of F-actin. In panel *D,* the addition of 30 μg F-actin to the *trans* compartment and the addition of 30 μg F-actin preincubated with cytochalasin B (Cyto B) (at a mass ratio of 5:1) to the *cis* compartment had similar effects on the gating of VDAC channels. Record shown was taken 20 min after the addition.

ing & Colombini, 1985) in order to gain insight into the mechanism by which actin may be modulating VDAC's voltage dependence. Analysis of the records prior to actin addition was not possible because these are kinetically delayed. The records after actin addition appear to have reached a steady state in the channel opening direction (declining electric field). The analysis of these records, assuming equilibrium between states, yielded 3 parameters: the steepness of the voltage dependence, n, the voltage representing the midpoint of the switching region, V_0, and the percentage drop in conductance in the closed state. The first two parameters were not very different from previously reported values: $n = 3.14 \pm 0.03$ (5) and $V_0 = 27 \pm 3$ mV (5). These are means ± SD (# of experiments). This result would indicate that actin is merely increasing the rates of the gating processes and not changing the inherent voltage-gating properties. However, a large and significant difference was observed in the large increase in the percent drop in conductance: 78 ± 4% (5). Typically the value is around 50% (Liu & Colombini, 1992) but with actin it was found to be as high as 85%. This result indicates that actin causes the channels to close to states of much lower permeability.

Actin addition to one side did not strictly affect only one of the 2 gating processes. Actin addition to one side primarily influenced the gating process elicited by applying negative potentials on the actin side. However, subsequent actin addition to the opposite side also influenced, to a small degree, the very same gating process. Note the small change in the negative current in Fig. 1*A* and compare the positive current records for panels *A* and *B*. This cross effect resulted in a small change in the voltage-dependence parameters. The extent of this cross effect was not studied in detail.

N. crassa VDAC Inhibits Actin's Effect on DNase-I Activity

To further study the binding of actin to *N. crassa* VDAC, we examined the enzymatic activity of DNase-I. Actin is known to be able to inhibit DNase-I (Lazarides & Lindberg, 1974). Our previous result showed that the presence of DNase-I eliminated actin's effect on VDAC gating. Thus, when VDAC binds actin, it should also weaken actin's inhibitory effect on DNase-I activity. To test this, we examined DNA hydrolysis by DNase-I in the presence of actin and VDAC proteins. The use of pure VDAC for these competition experiments was precluded by the relatively low concentration of protein and the fact that the associated detergent, Triton X100, inhibits DNase I activity. Therefore mitochondrial membranes containing VDAC proteins were used.

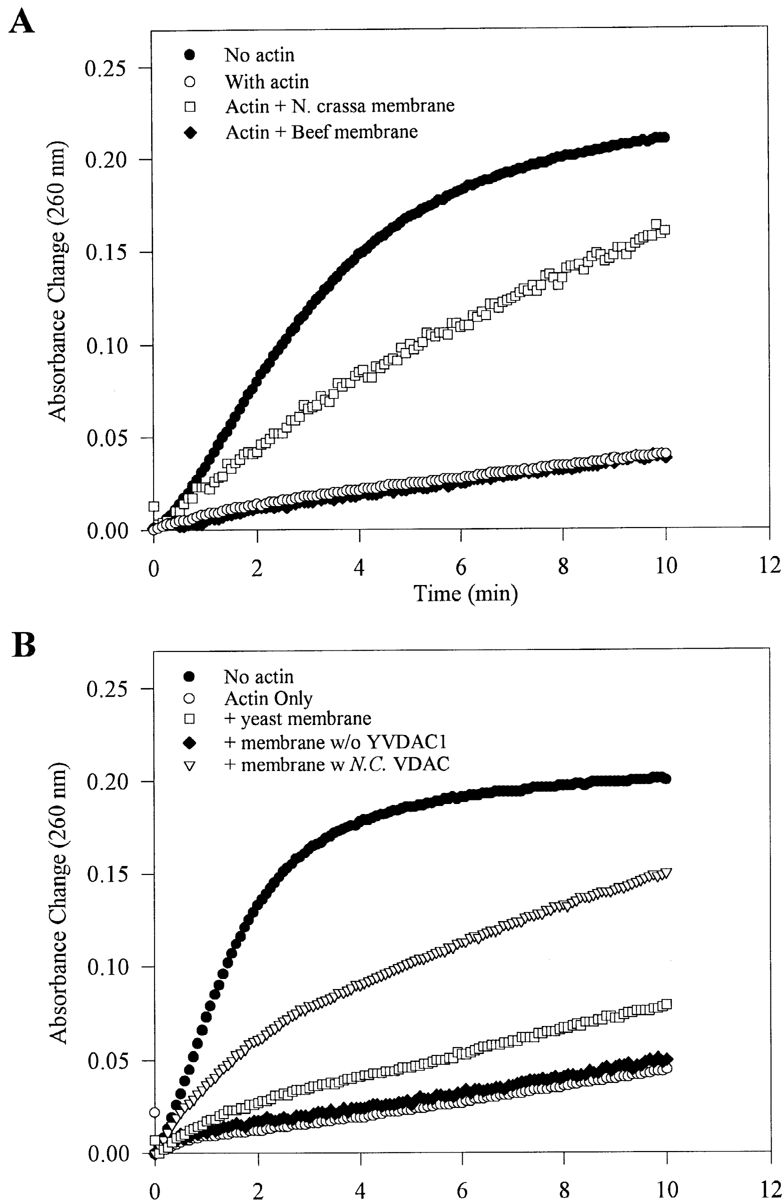

Fig. 4. DNase-I activity assay in the presence of actin and mitochondrial membrane containing different VDAC proteins. In panel *A*, no actin (●), 1.0 μg actin (○), 1.0 μg actin plus *N. crassa* mitochondrial membrane containing 50 μg protein (□), or 1.0 μg actin plus beef liver mitochondrial membrane containing 50 μg protein (♦) was mixed into the DNA solution before adding DNase-I. In panel *B*, no actin (●), 0.5 μg actin (○), 0.5 μg actin plus yeast mitochondrial membrane without YVDAC1 containing 70 μg protein (♦), 0.5 μg actin plus yeast mitochondrial membrane containing *N. crassa* VDAC and 70 μg protein in total (▽), or 0.5 μg actin plus wildtype yeast mitochondrial membrane containing 70 μg protein (□) was mixed into the DNA solution before adding DNase-I.

As shown in Fig. 4*A*, DNase-I digested DNA at a high rate, as indicated by a fast increase in light absorbance. The addition of actin greatly inhibited the digestion process. The presence of *N. crassa* membrane dramatically decreased actin's inhibitory effect on DNase-I, indicating that *N. crassa* VDAC binds actin reducing the free actin concentration and thus reducing the amount of actin bound to DNase-I. Mitochondrial membranes from beef liver were ineffective.

To verify that it was indeed the *N. crassa* VDAC that was causing the change on actin's effect, *N. crassa* VDAC was expressed in yeast missing VDAC1 (the only version of VDAC in yeast that forms channels). Mitochondrial membranes isolated from wild-type yeast resulted in a small reduction in actin inhibition of DNase I activity (Fig. 4*B*) while these membranes isolated from yeast strains lacking yeast VDAC1 were without effect. In sharp contrast, mitochondrial membranes isolated from yeast lacking yeast VDAC1 but expressing *N. crassa* VDAC showed a pronounced reduction of the actin inhibition (Fig. 4*B*). With the same genetic background, it is unlikely that any other protein in the outer membranes was responsible for the effect. The presence of the plasmid without the *N. crassa* VDAC gene had no effect. VDAC and DNase-I may share the same binding site on actin.

Discussion

VDAC channels close to various extents depending on the applied voltage and on the presence of any modulat-

ing substance. Voltage-dependent closure of VDAC typically involves a drop in conductance of about 50% and an inversion of ion selectivity. The channels normally do not close completely. The combination of the drop in conductance and selectivity inversion results in an effective barrier to significant organic metabolites such as succinate, citrate, phosphocreatine (Hodge & Colombini, 1997; Vander Heiden et al., 2000) and is impermeant to ATP (Rostovtseva & Colombini, 1996, 1997). However, at higher voltages (above 70 mV; Holden & Colombini, 1988) or in the presence of modulating substances such as König's polyanion (Colombini et al., 1987) and the VDAC modulator-containing fractions (Holden & Colombini, 1988)), the channels can achieve states of very low conductance. Actin results in closed states whose conductance is lower than normal as shown by an approximately 80 percent drop in conductance. It is not clear from our current studies whether this is due to the channels occupying a different less conductive closed state or whether the actin is partially blocking the closed state.

The measured steepness in the voltage dependence is very close to normal. This is important because actin protein has a net negative charge (Martonosi, Molino & Gergely, 1964) and polyanions are known to induce VDAC closure by increasing the steepness of the voltage dependence. This is true both for highly charged substances like dextran sulfate and highly charged proteins like pepsin (Colombini, Holden & Mangan, 1989). Since actin did not produce abnormally high steepness of the voltage dependence, it is not acting like other polyanions. It looks as if actin simply reduces some energy barrier to closure.

VDAC channels purified from *N. crassa* mitochondria and reconstituted into planar phospholipid membranes can respond to less than 50 nM α-actin. The actin affects the channels from either side of the membrane but from one side it mainly affects only one of the two gating processes. Actin favors channel closure when the actin side is made negative. A negative potential would draw the positively charged voltage sensor toward the actin side (Doring & Colombini, 1985; Mangan & Colombini, 1987). Thus, the ability of actin to favor closure in this way is consistent with our understanding of the voltage gating process in VDAC (Song et al., 1998).

That actin itself is responsible for the observed effects on VDAC channels is clear from a number of control experiments. The addition of pronase rapidly reverses the actin effect. Bovine serum albumin at doses that are over 2000 times those needed to observe effects with actin, is totally ineffective. DNase-I, a protein that binds tightly to actin (Kabsch et al., 1990), blocks the effect. Phalloidin, a toxin that specifically binds to F-actin, blocks the effect if it is preincubated with F-actin prior to addition. In view of these effects, it is unlikely that a contaminant, such as an actin-binding protein, would be responsible for the observed effect.

While F-actin might still bind, the results clearly indicate that it is G-actin that is responsible for modulating the voltage gating of VDAC. G-actin from two different sources (rabbit muscle and porcine heart muscle) works very well and it is virtually impossible for the G-actin added to spontaneously form F-actin under the conditions used. The addition of F-actin also works in increasing the ability of the VDAC channels to close, but it is likely the F-actin is depolymerizing in solution because the concentrations used (as low as 50 nM) are below the critical concentration (70–360 nM; Panaloni et al., 1984). When F-actin was stabilized by preincubation with phalloidin, it was no longer functional. Phalloidin reduces the critical concentration to 37 nM (Faulstich, Schafer & Weckauf, 1977) and this may be lower than the concentration needed to bind to VDAC. The small size of phalloidin would make it unlikely that it would physically interfere with the binding of F-actin to VDAC. Moreover, if phalloidin was added after actin had increased the voltage sensitivity of VDAC, it was ineffective. Thus phalloidin does not act by binding directly to VDAC.

The binding of DNase-I to actin blocked the ability of actin to bind to and affect VDAC's gating properties. In addition, the ability of actin to inhibit DNase I activity was reduced by the presence of VDAC. Thus DNase I and VDAC may be binding to the same site on actin. The binding of DNase I blocks only one portion of the actin molecule (Kabsch et al., 1990) and this may also be the VDAC binding site. If VDAC does bind at this site, the negatively charged regions of actin are left largely exposed but these are clearly not sufficient to affect VDAC in the same way as polyanions have been reported to act (Colombini et al., 1989; Mangan & Colombini, 1987). Of course, the bindings sites might differ and influence each other allosterically.

The small molecule, cytochalasin B, bound to actin but did not interfere with the ability of actin to modulate the voltage dependence of VDAC. Cytochalasin B severs F-actin into short filaments and it also binds to G-actin (Cooper, 1987; Urbanik & Ware, 1989). Thus G-actin molecules that have cytochalasin B bound still bind to VDAC. Presumably the small size of cytochalasin B does not produce any significant steric inhibition and so does not interfere with actin binding to VDAC.

The physiological role of actin binding to VDAC and modulating its voltage dependence is unclear. If mitochondria are to move along actin filaments, a motor protein should be involved in the attachment of VDAC to F-actin. Evidence for VDAC's involvement in the microtubule-based system for translocating mitochondria within the cell comes from evidence that VDAC binds MAPs (Linden & Karlsson, 1996). Other mitochondrial proteins have been identified as the ones that bind to actin filaments (Boldogh et al., 1998). Thus VDAC's ability to bind to and modulate G-actin may be related to regulation of energy metabolism. In a quiescent cell, the

higher free actin concentration might keep some of the VDAC channels closed resulting in lowered mitochondrial function. When a cell becomes activated and uses actin-based motility for cell locomotion or organelle transport, the cytosolic concentration of free G-actin may be reduced resulting in the dislocation of G-actin from VDAC and the opening of VDAC channels. This would increase the metabolic flux across the mitochondrial outer membrane and increase mitochondrial metabolism including ATP production. This possible regulatory process may have been lost with evolution since we found that actin had a weak and poorly reproducible effect on mammalian VDAC. On the other hand, the presence of different actins and actin-like proteins in mammalian cells may mean that alpha actin is no longer the protein that performs this function in mammalian cells.

It is also possible that actin binding to VDAC is the pertinent result. The fact is that binding of actin to *N. crassa* VDAC results in an augmentation of channel closure, which may be a secondary result arising from the proximity of negative charge on actin with VDAC's voltage sensor. The weak and poorly reproducible effect seen with mammalian VDAC may indicate that actin binds also to mammalian VDAC but does not have the same effect on the voltage gating and thus the binding is difficult to detect. When *N. crassa* VDAC was reconstituted into mixed soybean phospholipids rather than using diphytanoyl phosphatidylcholine, its voltage dependence was not significantly affected by added actin.

Actin is clearly not the elusive VDAC modulator. The VDAC modulator has been localized to the intermembrane space of mitochondria (Holden & Colombini, 1988), although there are reports of similar activity in the cytosol (Elkeles, Breiman & Zizi, 1997). The VDAC modulator has been shown to act on VDAC at a concentration that is about 1 order of magnitude lower (Liu et al., 1994). It increases the steepness of the voltage dependence by 2- to 3-fold (Liu & Colombini, 1992) and can induce VDAC channels to enter very low conducting states. It is highly conserved and can act on VDAC from all sources (Liu & Colombini, 1991). Partially purified preparations from potato mitochondria act on fungal and mammalian VDAC and vice versa. There may be a family of proteins that modulate the voltage dependence of VDAC, and actin is one of these.

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