Beticolins, Nonpeptidic, Polycyclic Molecules Produced by the Phytopathogenic Fungus *Cercospora beticola*, as a New Family of Ion Channel-Forming Toxins

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Beticolins are toxins produced by *Cercospora beticola*, a phytopathogenic fungus responsible for the leaf spot disease of sugar beet. They form a family of 20 nonpeptidic compounds (named B0 to B19) that share the same polycyclic skeleton but differ by isomeric configuration (*ortho*- or *para*-) and by a variable residue *R* (bridging two carbons in one of the six cycles). It has been previously shown that B0 assembles itself into a multimeric structure and forms ion channels into planar lipid bilayers (C. Goudet, A.-A. Véry, M.-L. Milat, M. Ildefonse, J.-B. Thibaud, H. Sentenac, and J.-P. Blein, *Plant J.* 14:359-364, 1998). In the present work, we investigate pore formation by three *ortho*-beticolins, B0, B2, and B4, and their related (i.e., same *R*) *para*-isomers, B13, B1, and B3, respectively, using planar lipid bilayers. All beticolins were able to form ion channels with multiple conductance states, although the type of cyclization (*ortho*- or *para*-) and residue (*R*) result in variations of channel conductance and ionic permeability, respectively. Channel formation by beticolins is likely to be involved in the biological activity of these toxins.

The genus *Cercospora* is a large group of fungal plant pathogens that cause disease worldwide on important crop species such as corn, coffee, tobacco, soybean, banana, and sugar beet. Due to their broad host range and worldwide distribution, *Cercospora* species are of major economic impact. *Cercospora beticola* Sacc. is the phytopathogenic fungus responsible for the leaf spot disease of sugar beet (*Beta vulgaris* L.). In vitro, this fungus produces a toxin named cercosporin and a family of other toxins named beticolins that are thought to be involved in its pathogenicity (Ballio 1991).

Cercosporin is a perylenequinone secondary metabolite (Lousberg et al. 1971; Yamazaki and Ogawa 1972) with a non-host-specific toxicity (Balis and Payne 1971; Fajola 1978; Daub and Ehrenshaft 1993). Toxic effects of cercosporin on plant cells are well documented. Toxicity is due to the pro-

duction of oxygen active forms by light-activated cercosporin (Daub 1982; Daub and Hangarter 1983; Dobrowolski and Foote 1983; Hartman et al. 1988), leading to membrane lipid peroxidation (Knox and Dodge 1985).

Beticolins form a family of 20 nonpeptidic compounds, named beticolin-0 to -19 (B0 to B19). They share the same polycyclic skeleton with a chlorine atom and partially hydrogenated anthraquinone and xanthone, but differ by isomeric configurations (*ortho*- or *para*-) and by a variable residue (*R*) bridging C11' and C14' (Fig. 1) (Milat et al. 1992; Prangé et al. 1997). Beticolins are non-host-specific phytotoxins that cause a broad range of cytotoxic effects. In planta, beticolins induce dramatic loss of solutes such as amino acids and β -cyanin from root tissues (Schlösser 1971; Macri and Vianello 1979). These non-host-specific toxins inhibit ATP-dependent H^+ transport in pea stem (Macri et al. 1983) and corn root (Blein et al. 1988). On tobacco cells, beticolins cause membrane depolarization (Gapillout et al. 1996). Biophysical studies showed that beticolins are able to chelate Mg^{2+} and to

Fig. 1. Structure of *ortho*- and *para*-beticolins.

form (B-Mg)₂ dimeric complexes (Mikes et al. 1994a). These complexes have a higher affinity for membranes than beticolin monomer (Mikes et al. 1994b; Gomès et al. 1996b). In addition to their effects on membrane transports, beticolins exhibit an antibiotic activity (Schlösser 1962). In vitro, beticolins also display an O₂⁻ scavenging activity (Rustérucchi et al. 1996) and an anti-proliferative effect on rat adrenocortical cell lines (Ding et al. 1996).

It was recently demonstrated that beticolin-0 molecules are able to assemble themselves in the presence of Mg²⁺ and to form weakly cationic selective channels that strongly increase membrane conductance (Goudet et al. 1998). In the present work, we investigate ion channel formation by six toxins of the beticolin family (B0, B13, B2, B1, B4, and B3) with planar lipid bilayer and voltage clamp techniques, which provide a powerful model (Kagan and Sokolov 1994). The chosen beticolins constitute three pairs of *ortho/para* isomers (Fig. 1). We demonstrate that any of these toxins can form ion channels displaying multiple conductance states. These channels are compared in terms of voltage dependence, conductance in different salt media, ionic selectivity, and Mg²⁺ sensitivity. The results are discussed in terms of the structure-activity relationship. It is likely that the deleterious properties of beticolins involve this pore-forming property, as demonstrated for syringomycin, a necrosis-inducing lipopeptide toxin secreted by the phytopathogenic bacteria *Pseudomonas syringae* (Hutchison et al. 1995; Feigin et al. 1996; Bender et al. 1999; Dalla Serra et al. 1999b).

RESULTS

Ion channel activity of beticolins.

A few minutes after addition of 10 µM beticolin (B0, B13, B1, B2, B4, or B3) to the *cis* medium, discrete current transi-

tions were recorded. Figure 2 shows examples of 12-s-long current records displaying different amplitude levels observed after addition of 10 µM B4 (Fig. 2A) or B3 (Fig. 2B) to the *cis* medium. Similar "single channel" events were observed for other forms of beticolin (not shown, but see Goudet et al. 1998 for B0). In standard solution, the free Mg²⁺ concentration was estimated to be 1 µM. When 50 µM MgCl₂ was added into the *cis* medium the beticolin-induced current increased and a macroscopic current could be recorded (not shown), as previously reported for B0 (Goudet et al. 1998). In the present study, we focused on unitary current analysis.

Multiple conductance values of beticolin channels.

In conditions allowing recording of unitary current (i.e., 1 µM Mg²⁺), all beticolin channels displayed at least three conducting states. For any beticolin, current traces similar to those shown in Figure 2 were obtained at different voltages from -120 to +120 mV, allowing current-voltage (i-V) relationships of the first three conducting levels formed by each of these toxins to be determined (Fig. 3). The relationships are all approximately linear and cross the axes close to the origin. Data in Figure 3 are from records of experiments with symmetrical *cis/trans* 200 mM NaCl standard solutions. Similar results were obtained when NaCl was replaced by either KCl or LiCl (not shown, but see Table 1). Numeric values of conductance for the first three conducting levels of each studied beticolin are given in Table 1. Whatever the beticolin and the ionic conditions, the conductance of level three was approximately twice that of level two, itself being approximately twice that of level one. Whatever the *ortho/para* isomer pair considered, the conductance induced by the *para*-isomer was 20 to 30% higher than that induced by the *ortho*-isomer. The B0/B13 pair displayed lower conductance than the B4/B3 pair, which itself produced less conductance than the B2/B1 pair.

Fig. 2. Unitary currents of different amplitudes induced by beticolins through a planar lipid bilayer. Representative trace of channel activity recorded at +120 mV in standard symmetric solutions a few minutes after addition of 10 µM concentrations of (A) *ortho*-B4 or (B) *para*-B3 toxin to the *cis* solution. Both *cis* and *trans* standard solutions contained 200 mM NaCl, 0.44 mM MgCl₂, 1 mM EDTA, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.4). The free Mg²⁺ concentration was estimated to be 1 µM. Data were recorded at 2 kHz and filtered at 50 Hz. Current of the closed (0) state is indicated by the solid line. Dotted lines (marked by 1, 2, and 3) show the three different discrete current amplitude levels determined from an amplitude histogram.

Conductance of beticolin-formed channels thus appeared to depend on both the type of isomerization and the type of R residue.

Ionic selectivity.

The cation/anion selectivity of beticolin channels was determined by measuring the current reversal potential (E_{rev}) in 500 mM/100 mM (*cis/trans*) concentration gradients of chloride salts (KCl, NaCl, or LiCl). In these conditions, the equilibrium potentials of Cl^- ions and that of the accompanying cation were close to -40 and $+40$ mV respectively. For each

tested beticolin, E_{rev} values slightly depended on the chloride salt used, revealing some ionic selectivity. However, in a given salt gradient, the values were roughly the same for the first three conductance levels (exact individual values were found within a 3-mV range around the average value). E_{rev} values were positive, indicating selectivity for cations versus Cl^- . From these data, P_X/P_{Cl} permeability ratios and thereby P_X/P_K ratios (X being Na, Li, or K) were derived with the Goldman-Hodgkin-Katz equation (Table 2). Except for B4 and B3, all the P_X/P_K and G_X/G_K ratio values were <1 . Thus, B4 and B3 showed a slight selectivity for Na^+ or Li^+ versus K^+ ,

Fig. 3. Current-voltage relationship of *ortho*-beticolins B0, B2, and B4 and corresponding *para*-beticolins B13, B1, and B3. Current amplitude of level 1, 2, and 3 is represented by ■, ●, ▲, respectively. Amplitude values of the current (i ; mean value; $n \geq 3$ experiments) for each beticolin are plotted against the voltage value (V) at which they have been recorded. Beticolin concentration was 10 μ M. Both *cis* and *trans* side medium contained 200 mM NaCl, 0.44 mM $MgCl_2$, 1 mM EDTA, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.4). Free Mg^{2+} concentration was estimated to be 1 μ M.

while the other studied beticolins showed a slight selectivity for K⁺ versus Na⁺ and Li⁺.

DISCUSSION

A new family of ion channel-forming toxins.

Addition of 10 μ M concentrations of beticolins (B0, B13, B2, B1, B4, or B3) induced discrete current transitions, similar to those occurring upon opening and closing of ion channels, indicating that all six beticolins are able to form ion channels, as previously demonstrated for B0 (Goudet et al. 1998).

It is therefore likely that all beticolins are able to form ion channels, due to the relative similarity of their structure (Ducrot et al. 1994b, 1994a; Prangé et al. 1995, 1997). X-ray diffraction studies of beticolin microcrystals allowed the determination of the 3-D structure of the molecule (Prangé et al. 1997). Beticolin molecules are no more than 17 Å long (Fig. 1) and, therefore, a multimeric assembly is likely to be necessary to form a pore across a 30-Å-thick lipid bilayer. It has been shown that, in the presence of Mg²⁺, a stable and electrically neutral dimer assembles that is composed of two beticolin molecules and two Mg²⁺ ions (Gomès et al. 1996a). Interestingly, this association with Mg²⁺ strongly increases partitioning of the toxin into the hydrophobic phase (Mikes et al. 1994b). The 3-D structure of the complex composed of two B1 molecules and two Mg²⁺ ions was elucidated by Jalal et al.

(1992). As previously hypothesized for B0 (Goudet et al. 1998), (B-Mg)₂ dimers would be the basic element of the pore.

Common features between beticolin channels.

A first common feature of beticolin channels is their poor ionic selectivity. Reversal potentials were determined in asymmetrical solutions of KCl, NaCl, or LiCl. The permeability ratios derived from these data with the Goldman-Hodgkin-Katz equation (Table 2) indicated that beticolin channels were poorly selective, as previously reported for B0 channels (Goudet et al. 1998). Cations like K⁺, Na⁺, or Li⁺ are more permeable than Cl⁻ but all these ions have a significant permeability through beticolin channels (Tables 1 and 2).

A second common feature of beticolin channels is their linear current-voltage relationships, whatever the conductance level considered (Fig. 3). The slope conductance is therefore independent of voltage. Ions go through the pore at a rate that is simply proportional to the driving force, and as easily in both directions. These data indicate that (i) channel formation/operation is poorly voltage sensitive and (ii) no intrinsic rectification is enabled as a result of the symmetrical pore structure.

A third common feature of beticolins is their ability to form channels displaying multiple conductance levels. The conductance values derived for the first three conducting states are in a geometric progression of ratio 2: conductance of state

Table 1. Conductance of *ortho*- and *para*-beticolins into 200 mM NaCl, KCl, or LiCl medium

Conductance in different media ^a	<i>ortho</i> -beticolins			<i>para</i> -beticolins		
	B0	B2	B4	B13	B1	B3
γ NaCl						
Level 1	8 $\pm$ 0.5 (5)	21 $\pm$ 1 (3)	11 $\pm$ 0.5 (3)	13 $\pm$ 1 (4)	29 $\pm$ 1 (3)	17 $\pm$ 1 (5)
Level 2	17 $\pm$ 1 (5)	43 $\pm$ 2 (3)	24 $\pm$ 1 (3)	26 $\pm$ 2 (4)	51 $\pm$ 2 (3)	34 $\pm$ 1 (5)
Level 3	29 $\pm$ 1 (5)	77 $\pm$ 4 (3)	48 $\pm$ 2 (3)	49 $\pm$ 3 (4)	95 $\pm$ 5 (3)	60 $\pm$ 2 (5)
γ KCl						
Level 1	11 $\pm$ 0.5 (5)	23 $\pm$ 1 (3)	11 $\pm$ 0.5 (3)	15 $\pm$ 2 (5)	28 $\pm$ 1 (3)	16 $\pm$ 1 (4)
Level 2	23 $\pm$ 1 (5)	41 $\pm$ 1 (3)	21 $\pm$ 1 (3)	31 $\pm$ 4 (5)	56 $\pm$ 2 (3)	32 $\pm$ 2 (4)
Level 3	47 $\pm$ 2 (5)	79 $\pm$ 4 (3)	41 $\pm$ 2 (3)	63 $\pm$ 7 (5)	118 $\pm$ 4 (3)	57 $\pm$ 2 (4)
γ LiCl						
Level 1	ND ^b	18 $\pm$ 2 (2)	11 $\pm$ 0.5 (3)	ND	24 $\pm$ 1 (3)	16 $\pm$ 1 (4)
Level 2	ND	36 $\pm$ 4 (2)	25 $\pm$ 1 (3)	ND	40 $\pm$ 2 (3)	31 $\pm$ 1 (4)
Level 3	ND	70 $\pm$ 7 (2)	49 $\pm$ 2 (3)	ND	80 $\pm$ 2 (3)	58 $\pm$ 2 (4)

^a Conductance values are expressed in pS. Values are mean $\pm$ SE (*n* experiments) measured in symmetrical solutions containing 200 mM NaCl, KCl, or LiCl, 0.44 mM MgCl₂, 1 mM EDTA, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (free Mg²⁺ concentration being estimated to be 1 μ M).

^b Not determined.

Table 2. Ionic selectivity of beticolins channels

Permeability ^a and conductance ^b ratio	<i>ortho</i> -beticolins			<i>para</i> -beticolins		
	B0	B2	B4	B13	B1	B3
PNa/PK	0.65 $\pm$ 0.03 (3)	0.91 $\pm$ 0.01 (3)	2.14 $\pm$ 0.06 (4)	ND ^c	0.49 $\pm$ 0.05 (2)	2.77 $\pm$ 0.16 (4)
PLi/PK	ND	0.77 $\pm$ 0.01 (2)	1.93 $\pm$ 0.06 (3)	ND	0.33 $\pm$ 0.01 (2)	2.21 $\pm$ 0.05 (4)
GNa/GK	0.68 $\pm$ 0.05 (5)	0.97 $\pm$ 0.05 (3)	1.12 $\pm$ 0.04 (3)	0.84 $\pm$ 0.04 (4)	0.91 $\pm$ 0.09 (3)	1.07 $\pm$ 0.01 (4)
GLi/GK	ND	0.85 $\pm$ 0.05 (2)	1.12 $\pm$ 0.07 (3)	ND	0.74 $\pm$ 0.06 (3)	1.02 $\pm$ 0.02 (4)

^a Permeability ratios were deduced from reversal potential measured in asymmetric media with the Goldman-Hodgkin-Katz equation (Hille 1992). *trans* medium contained 100 mM XCl (where X = K, Na, or Li) whereas *cis* medium contained 500 mM XCl. Both *cis* and *trans* medium contained 0.44 mM MgCl₂, 1 mM EDTA, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-XOH (pH 7.4). The free Mg²⁺ concentration was estimated to be 1 μ M. Beticolin concentration was 10 μ M. Data are mean $\pm$ SE (*n* experiments).

^b Conductance ratios were calculated from the data in Table 1. Data are mean $\pm$ SE (*n* experiments). For conductance determination, both *cis* and *trans* solutions contained 200 mM XCl (where X = K, Na, or Li; see legend to Table 1).

^c Not determined.

two is twice the conductance of state one and conductance of state three is twice the conductance of state two (Table 1). A fourth conductance state is less often observed. This higher conducting state follows this progression (not shown). Two main hypotheses could be envisaged concerning the type of channel structures underlying such a behavior (Meves and Nagy 1989). The first hypothesis is a cluster organization of beticolin elementary channels that would open and close simultaneously, as proposed to explain the multiple conductance states of channels formed by syringomycin E (Kaulin et al. 1998). The second hypothesis is that multiple conductance states of beticolins result from different channel structures, as proposed for channels formed by alamethicin (Bezrukov and Vodyanoy 1993; Vodyanoy et al. 1993). In the case of beticolin channels, the fact that the first three conductance levels display similar ionic selectivity suggests similar pore structure and size, as this would be the case within clusters. With non-electrolytes of different hydrodynamic sizes, we have demonstrated that the same pore size (pore radius approximately 7.5 Å) underlies the first three conductance levels of B3 channels and thereby we have supported the cluster hypothesis (Goudet et al., *in press*). As *ortho*-beticolin and *para*-beticolin channels display similar structural and functional properties, it can be assumed that cluster organization is a common feature of beticolin channels.

Finally, whatever the form of beticolin present at a concentration of 10 µM, macroscopic currents could be recorded in the presence of high Mg²⁺ concentration (data not shown, but see Goudet et al. 1998). In the present study, low free Mg²⁺ concentration (1 µM) was used to record reproducibly single channel rather than macroscopic currents. It is therefore concluded that Mg²⁺ sensitivity is a feature common to all beticolins.

Differences between beticolin channels.

Although beticolins have similar structures, they differ by the type of isomerization and/or the *R* residue bridging C11' and C14' of the common skeleton (Fig. 1). Since all beticolins seem to form channels with common features, it is worth (i) noting that some functional features differ between channels formed by different beticolins and (ii) discussing how these differences are related to the structural variations in the beticolin family.

As shown in Table 1, whatever the *ortho/para* pair considered, the *para*-isomer forms channels with a higher (20 to 30%) conductance than that of the channels formed by the *ortho*-isomer. As discussed above, the (B-Mg)₂ dimer is likely to be the basic element forming beticolin channels. The dimer structure forms a trapezoidal frame (Jalal et al. 1992). Here we propose that the hollow at the center of the frame would form the pathway for ions through the structure. From what is known about the molecular shape of *ortho*- and *para*-beticolins (Prangé et al. 1997) it can be proposed that *para*-beticolin dimers make a larger pore than those formed by *ortho*-beticolin dimers, thus resulting in ion channels with larger conductance.

Despite the fact that *R* is not believed to line the pore, it is likely to influence the dimer shape and thereby the pore structure. Our data show that the conductance and the ionic selectivity of beticolin channels depend on *R*. For example, B2/B1 form channels with a higher conductance (i.e., pore size) than the others studied (Table 1) and B4/B3 form chan-

nels with a different selectivity sequence than the other beticolins studied.

Biological significance.

A pore radius in the range of 7.5 Å is likely to enable permeation of sugars and amino acids through the plasmic membrane. Ion channel formation by beticolins is thus consistent with previous data indicating that, in plant cells, beticolins induced loss of electrolytes, amino acids, and betacyanine (Macri and Vianello 1979) and depolarized transmembrane potential (Gapillout et al. 1996). Inducing permeabilization of cell membranes is one of the most common killing mechanisms of cytotoxic molecules. Pore formation has been suggested to be responsible for membrane permeabilization, collapse of ionic and electrical gradients, and cell lysis for several peptidic or nonpeptidic toxins or antibiotics: e.g., gramicidin (Woolley and Wallace 1992), alamethicin (Woolley and Wallace 1992; Sansom 1993), phallolysin (Wilmsen et al. 1985), flammutoxin (Tomita et al. 1998), lycotoxin (Yan and Adams 1998), and yeast killer toxins (Kagan 1983). As demonstrated, for example, for necrosis-inducing lipopeptide toxins secreted by the phytopathogenic bacteria *Pseudomonas syringae* (Hutchison et al. 1995; Feigin et al. 1996; Hutchison and Gross 1997; Bender et al. 1999; Dalla Serra et al. 1999a, 1999b), the ability of beticolins to form ion channels is therefore likely to be involved in the biological activity of these toxins.

MATERIAL AND METHODS

Beticolins.

Beticolins were extracted from the mycelium of the *C. beticola* strain CM and purified as previously described (see Ducrot et al. 1996 and references therein).

Planar lipid bilayer.

Planar bilayers were formed across a 0.2-mm-diameter hole made in PVDF (polyvinylidene fluoride) septum separating two chambers of 2 ml (defined as *cis* and *trans*) by painting a solution of synthetic POPE and POPC (1-palmitoyl-2-oleoyl-phosphatidylethanolamine and 1-palmitoyl-2-oleoyl-phosphatidylcholine, respectively; Avanti Polar Lipids, Birmingham, AL) in a 1/1 molar ratio dissolved in *n*-decane (10 mg · ml⁻¹; Sigma, St. Louis, MO) (Mueller et al. 1962).

trans and *cis* chamber solutions were connected (through 3 M KCl/AgCl/Ag half-cells) to headstage and signal-ground inputs, respectively, of an Axopatch 200B (Axon Instrument, Foster City, CA) patch-clamp amplifier. Potential values were thus defined as the voltage of *trans* chamber minus the voltage of *cis* chamber. Membrane capacitance and resistance monitoring were used to verify membrane formation. Bilayers readily formed with a capacitance of 100 to 150 pF. For a period of at least 30 min prior to toxin addition, those membranes were stable and, in standard solution, had a conductance less than 10 pS at voltages in the ±100 mV range. Recordings were sampled at 2 kHz and filtered at 50 Hz for further analysis with pClamp 6 (Axon Instrument) and Origin 4.0 (Microcal Software, Northampton, MA) software. To determine unitary amplitude values at a given voltage, amplitude histograms were done and analyzed (Sakhman and Neher 1995). To determine slope conductance (γ), these amplitude

values were plotted against voltage, and conductance was then derived from the slope of current-voltage relationships.

Solutions.

Standard solutions contained 200 mM XCl (with X = K, Na or Li), 0.44 mM MgCl₂, 1 mM EDTA, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.4). The free Mg²⁺ concentration was estimated to be 1 μM. In standard experiments, both the *cis* and *trans* chambers contained the above solution.

Asymmetric solutions were used in order to investigate ionic selectivity of beticolin channels. *trans* medium contained 100 mM XCl (with X = K, Na, or Li) whereas *cis* medium contained 500 mM XCl, the other concentrations being unchanged. Reversal potentials (*E*_{rev}) in these asymmetric solutions were measured and the cation/anion permeability ratios were then calculated from the *E*_{rev} values with the Goldman-Hodgkin-Katz equation (Hille 1992).

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