MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 4, pp. 407-411, 1991

Research Notes

Bacterial Blotch Disease of the Cultivated Mushroom Is Caused by an Ion Channel Forming Lipodepsipeptide Toxin

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Received 13 November 1990. Accepted 8 February 1991.

Tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii* was shown to form voltage-dependent, cation-selective, ion channels in planar lipid bilayers. Tolaasin-induced ion channels were inhibited by the addition of Zn^{2+}, which also inhibited the activity of tolaasin on horse erythrocytes and mushroom tissue. Tolaasin-144, an altered form of tolaasin lacking three consecutive D-amino acids from the α-helical portion of the molecule, did not form ion channels in planar lipid bilayers, did not lyse erythrocytes, and showed reduced ability to cause disease symptoms on mushroom tissue.

Additional keyword: Agaricus bisporus.

Pseudomonas tolaasii Paine, causal organism of the economically significant brown blotch disease of the cultivated mushroom *Agaricus bisporus* (Lange) Imbach (Tolaas 1915), produces an extracellular toxin (Nair and Fahy 1973; Peng 1986). The structure of the toxin, tolaasin, has been determined (Nutkins *et al.*, in press) and is a lipodepsipeptide (MW = 1,985) consisting of 18 amino acid residues with a β-octanoic acid group at the N-terminus. Recent studies have shown that this toxin, tolaasin, is solely responsible for disease symptoms on mushrooms and that it is able to disrupt the membranes of fungal, bacterial, plant, and animal cells (Rainey *et al.*, in press). Divalent metal ions and high molecular weight sugars have been shown to inhibit tolaasin-induced hemolysis of erythrocytes (Rainey *et al.*, in press), which suggests that tolaasin, like some other cytotoxic agents, forms discrete pores in erythrocyte membranes (Pasternak 1986; Bashford *et al.* 1986), and that lysis occurs via a colloid osmotic mechanism (Knowles and Ellar 1987; Weiner *et al.* 1981). These results, combined with ultrastructural studies of blotched and toxin-treated mushrooms (Cole and Skellerup 1986; Rainey *et al.*, in press), firmly implicate cell membranes as primary sites of tolaasin action.

Many membrane-bound and membrane-active proteins contain regions of amphipathic α-helix that associate within the membrane, producing structures that are internally hydrophilic and externally hydrophobic, for example, bacteriorhodopsin (Engelman *et al.* 1980) and the *Staphylococcus aureus* Rosenbach α-toxin (Fussle *et al.* 1981). A number of small, membrane-active peptides also have regions of amphipathic α-helix and aggregate to form pores in membranes. Among these are the ion channel forming peptides alamethicin (Fox and Richards 1982), melittin (Lauterwein *et al.* 1979), and gramicidin A (Weinstein *et al.* 1985). Studies on the three-dimensional structure of tolaasin in sodium dodecyl sulfate (SDS) solution have shown that it also contains an amphipathic α-helical region (Mortishire-Smith *et al.*, 1991), further suggesting that tolaasin may cause cellular disruption via the formation of protein pores in membranes in a manner similar to that of other membrane-active peptides.

This work concerns the ion channel forming properties of tolaasin and of a structural analogue of tolaasin (tolaasin-144) that lacks three amino acids from the α-helical region of the molecule (Nutkins *et al.*, in press).

Strains. *P. tolaasii* NCPPB 1116 was obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, U. K. *P. tolaasii* PT144 was generated from NCPPB 1116 by transposon Tn*5* mutagenesis (Simon *et al.* 1983). Tolaasin-defective mutants were detected by using the 'white line in agar test' (Wong and Preece 1979). PT144 produces a white line of reduced intensity compared with the wild-type strain (Nutkins *et al.*, in press).

Isolation and purification of tolaasin. Tolaasin and tolaasin-144 were isolated from culture filtrates of *P. tolaasii* strains NCPPB 1116 and PT144 and purified by reverse-phase high-performance liquid chromatography (HPLC) with an S5P (phenyl) column (Nutkins *et al.*, in press). Tolaasin and tolaasin-144 were each collected from a single HPLC peak (Nutkins *et al.*, in press)

Lipid bilayers. Planar lipid bilayers were painted across a 0.2-μm-diameter hole in styrene copolymer, from a decane dispersion of 30 mM synthetic 1-palmitoyl-2-oleoyl phosphatidylethanolamine, to form a neutral bilayer (Knowles *et al.* 1989). Lipids were supplied by Avanti Polar Lipids, Birmingham, AL. The bilayer separated solutions of 300 μl of 100 or 500 mM KCl in a styrene copolymer cup (*cis* side) from 5 ml of 100 mM KCl in an outer Perspex

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chamber (*trans* side). Aqueous solutions were buffered with 5 mM *N*-tris-(hydroxymethyl)methyl-2-amino ethanesulfonic acid/*N*-methyl-D-glucamine, pH 7.5. All solutions were filter sterilized with a 0.2-μm filter (Sartorius Filters, Inc.) and experiments were carried out at room temperature.

Each bilayer was maintained either with an ionic gradient (500 mM KCl on the *cis* side, 100 mM KCl on the *trans* side), or in symmetrical 100 mM KCl for up to 1 hr (and never less than 15 min) to check for the presence of artefactual channel activity. If no channel activity was detected, tolaasin was added to the *cis* side of the bilayer and the contents of the cup were stirred.

The effect of Zn^{2+} ($ZnCl_2$) on ion channel activity was determined by adding the metal ion to the *cis* side of the bilayer to final concentrations of 10, 100, and 1,000 μM.

Current was monitored under voltage clamp conditions by using a low-noise operational amplifier with frequency compensation (Miller 1982), and data were stored unfiltered on videotape after being digitized by a Sony audio to digital converter (PCM 701 ES, 22 kHz per channel). Potential differences were recorded *cis* with respect to *trans*, the latter being held at ground. Traces were analyzed with a microcomputer-based system (patch clamp analysis software, Cambridge Electronic Design, Cambridge, U.K.) after digitization at 5 kHz and low-pass filtering at 100 Hz.

Biological assays. The hemolytic properties of tolaasin-144 were assessed with an erythrocyte lysis assay (Rainey *et al.*, in press). The activity of tolaasin and tolaasin-144 on both cut and intact mushroom cap tissue was determined as described by Rainey *et al.* (in press). The effect of Zn^{2+} on tolaasin activity was determined by adding $ZnCl_2$ to a final concentration of 10 mM (which caused no significant pH change).

Experiments carried out with HPLC-purified wild-type tolaasin resulted in the formation of ion channels across a previously high-resistance planar lipid bilayer. The addition of 0.2 μg (~0.3 μM) of tolaasin to the *cis* side of the voltage clamped membrane resulted in current jumps (within 10–20 sec) representing the movement of ions across the membrane. The toxin-generated channels showed very fast flickerings of current. Opening and closing of channels appeared to be cooperative as current flow alternated between periods of low activity and bursts of high activity (Fig. 1). It was not possible to follow the conductance of single ion channels because activity depended on a threshold concentration (0.3 μM) of tolaasin, which resulted in the incorporation of many channels into the membrane. Furthermore, it was difficult to incorporate the same number of channels into different bilayers, making it difficult to compare results from separate experiments in a quantitative manner. Variability in channel activity between bilayers was probably caused by incomplete partitioning of tolaasin into the bilayer caused by the unavoidable presence of excess lipid that forms an annulus around the bilayer. After appropriate digitization, macroscopic conductance data were, therefore, accumulated and analyzed by computer.

Tolaasin-induced conductance was voltage dependent (Fig. 2) and showed large increases in conductance only when the potential applied to the *cis* side (the side to which tolaasin was added) was positive. Experiments carried out in asymmetrical KCl (500 mM *cis* side, 100 mM *trans* side) showed the reversal potential for channel activity to be consistent with a high selectivity of the channels for K^+ over Cl^-, indicating that the channels are cation selective.

Addition of $ZnCl_2$ (at concentrations between 10 and 1,000 μM) to the *cis* side of the bilayer greatly reduced channel activity at all voltages tested. This effect was shown to be at least partially reversible if the $ZnCl_2$ was removed from the cup by perfusion. Quantitative comparison of data obtained from different bilayers was not possible due to the variable number of channels incorporated in separate experiments. However, the amount of Zn^{2+} required for complete inhibition of channel activity was directly proportional to the amount of activity observed, and an increase in the Zn^{2+} concentration was always accompanied by a decrease in membrane conductance (Fig. 3).

HPLC-purified tolaasin-144 showed no ability to form ion channels in planar lipid bilayers. The addition of up to 4 μg of this peptide (~8 μM) repeatedly showed no channel activity during 30 min of continuous observation. The addition of 0.2 μg (~0.3 μM) of wild-type tolaasin to the same bilayer after 30 min resulted in the movement of ions within 10–20 sec. Each of five replicate experiments was concluded in this way, indicating that the lack of channel activity was due solely to the inability of tolaasin-144 to form ion channels.

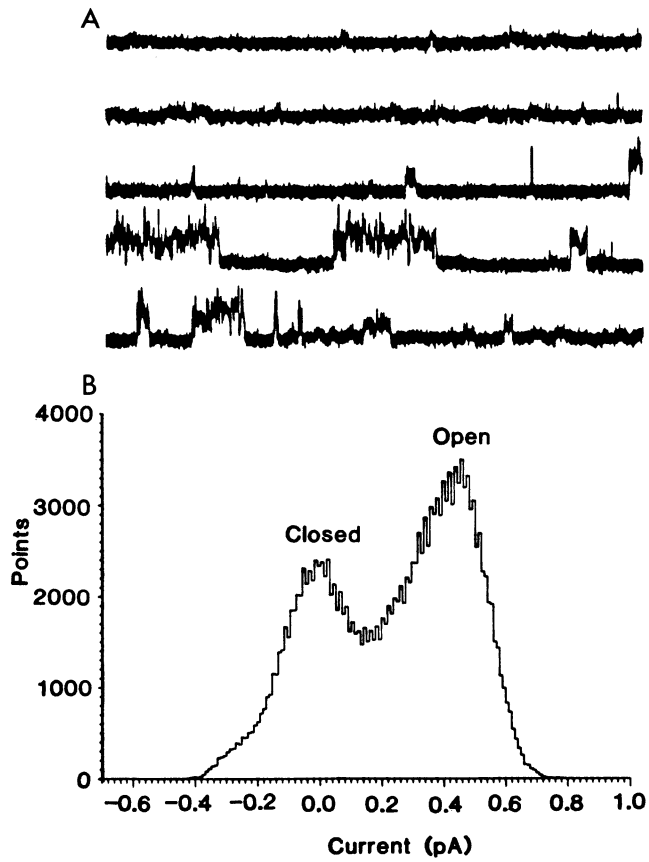

Fig. 1. A, Membrane current fluctuations induced by tolaasin in a planar lipid bilayer when clamped at +20 mV in 500:100 mM KCl. Openings are represented by upward deflections and the trace represents 45 sec of continuous recording, starting at the top left hand corner. **B,** Amplitude distribution for the above trace. Data were filtered at 100 Hz, digitized at 5 kHz and bin sizes represent 10 fA.

Tolaasin-144 failed to cause lysis of erythrocytes even at concentrations of up to 50 $\mu g\ ml^{-1}$, whereas rapid hemolysis was observed with 1–1.5 $\mu g\ ml^{-1}$ wild-type tolaasin (Rainey *et al.*, in press). The addition of 0.3 μg of tolaasin to the cut surface of a mushroom resulted in pitting of the exposed surface within 10 min. Concomitant addition of 10 mM $ZnCl_2$ with 0.3 μg of tolaasin resulted in total inhibition of pitting. Tolaasin-144 failed to pit cut mushroom tissue when added at levels comparable to the wild-type toxin, but slight pitting was observed when added at 20 μg (Table 1). When applied to intact mushroom caps, 0.15 μg of tolaasin caused browning and pitting after 16 hr. Concomitant addition of 10 mM $ZnCl_2$ eliminated the activity of 0.15 μg of tolaasin on intact caps. Tolaasin-144 was active on intact mushroom caps; however, its activity was reduced in comparison with native tolaasin (Table 1).

This work demonstrates the ability of tolaasin to form ion channels in planar lipid bilayers. The channels formed were highly voltage dependent, showing a steep rise in activity only when a *cis* positive potential was applied. Strong cation selectivity and burstlike characteristics were also noted. Similar studies of macroscopic conductance in planar lipid bilayers have been performed on a number of other small peptides such as nisin (Sahl *et al.* 1987), Pep5 (Kordel *et al.* 1988), alamethicin (Boheim 1974), melittin (Tosteson and Tosteson 1981), and defensins (Kagan *et al.* 1990). A *trans* negative (*cis* positive) potential is a requirement for the activity of all of these peptides and has obvious physiological significance because most cells have a potential difference across the plasma membrane such that the inside is negative with respect to the outside.

A high power dependence of activity on concentration at a fixed voltage has been demonstrated for melittin (Tosteson and Tosteson 1981) and alamethicin (Balasubramanian *et al.* 1981). This led to the suggestion that the channels formed by these peptides were the result of specific aggregations of monomers in the bilayer. Data that indicate the existence of single pores with multiple conductance states support this theory and have been obtained for alamethicin, Pep5, and nisin (Boheim 1974; Kordel *et al.* 1988 and Sahl *et al.* 1987). Tolaasin-induced ion channel activity showed a high dependence on toxin concentration; additions of tolaasin at concentrations of up to 0.2 μM had no effect on membrane conductance, while additions of 0.3 μM led to large increases in conductance. This demonstrated a definite threshold concentration for activity of tolaasin that is consistent with the association of monomers to produce single channels. Furthermore, the small size of tolaasin (MW = 1,985) makes it unlikely that a single molecule could be responsible for the formation of an ion channel across the membrane, and Rainey *et al.* (in press) also described a similar threshold effect for tolaasin activity on erythrocytes.

Many models have been proposed to explain the observed structural and functional properties of ion channels formed by small peptides. Alamethicin has been intensively studied and its activity is thought to depend on the aggregation of amphipathic α-helical monomers (Boheim 1974; Fox and Richards 1982; Mathew and Balarum 1983). The aggregation model proposed by Boheim (1974) suggests that the gain, or loss, of single monomers from the functional channel accounts for the multiple conductance states observed, whereas the helix dipole model of Mathew and Balarum (1983) considers the helix dipole moment of the alamethicin monomers to be the major factor modulating channel size, selectivity, and voltage dependence. Fox and Richards (1982) presented crystallographic data that confirmed the association of amphipathic α-helices and enabled them to postulate mechanisms to explain channel properties based on the observed molecular conformations. Tolaasin exhibits many structural and functional similarities with alamethicin, including a region of amphipathic α-helix, and may aggregate to form ion channels in a similar manner.

The inhibitory effect of Zn^{2+} on ion channel formation by a number of different peptides, including melittin, has

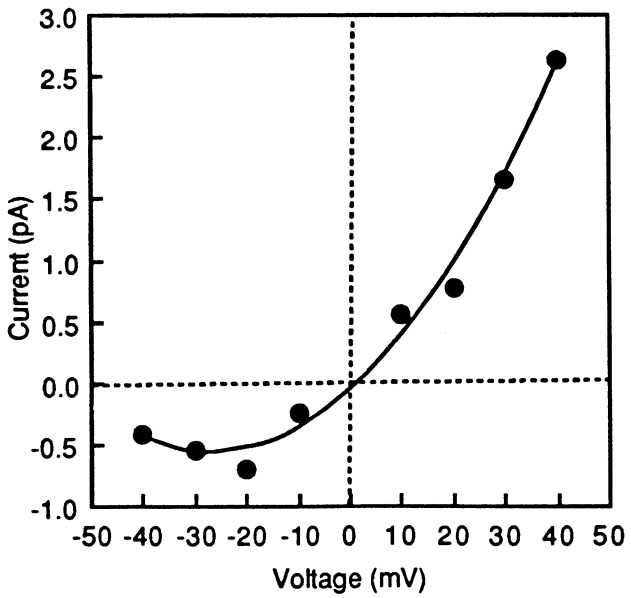

Fig. 2. Voltage dependence of channel opening. Plot of modal current through a large number of channels formed by tolaasin in a planar lipid bilayer against voltage in symmetrical 100 mM KCl.

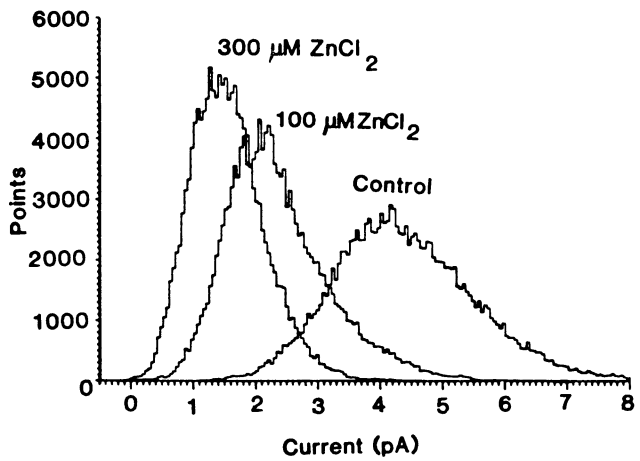

Fig. 3. Effect of Zn^{2+} on membrane current fluctuations induced by tolaasin in a planar lipid bilayer when clamped at +20 mV in 500:100 mM KCl. Plot shows amplitude distribution and data were filtered at 100 Hz, digitized at 5 kHz. Bin sizes represent 10 fA.

Table 1. Lowest detectable level of tolaasin and tolaasin-144 required to affect cut and intact mushroom tissue, erythrocytes, and planar lipid bilayers in the presence (+) and absence (−) of Zn^{2+} (10 mM)

	Tolaasin (μg)		Tolaasin-144 (μg)	
	−	+	−	+
Ion channels	0.2 (0.3 μM)	...[a]	...	...
Hemolysis	3.0[b] (0.25 μM)	...	...	...
Cut mushroom caps	0.3	1.25	20	...
Intact mushroom caps	0.15	0.3	0.6	...

[a]No activity.
[b]Result from P. B. Rainey *et al.*, in press.

been previously reported (Bashford *et al.* 1986; Pasternak 1986) and may occur via chelation of ionizable groups in the membrane near the site of pore formation. The complete inhibition of tolaasin-induced conductance in lipid bilayers by Zn^{2+}, combined with the ability of Zn^{2+} to prevent hemolysis of erythrocytes (Rainey *et al.*, in press) and to reduce damage to mushroom tissue, suggests that *de novo* formation of ion channels has a role in toxin activity.

The inability of the altered toxin, tolaasin-144, to form ion channels or to cause hemolysis of erythrocytes further implicates ion channel formation as the mechanism of hemolysis. Tolaasin-144 also caused negligible damage to cut basidiocarps (even at levels 1,000-fold higher than required for the wild-type toxin) but was only slightly reduced in its effectiveness on intact mushroom tissue. This observation highlights a clear difference between the action of tolaasin-144 on the cap surface and in the other systems tested and may be relevant to understanding the activity of wild-type tolaasin *in vivo*. If, as in the case of alamethicin, the α-helical portion of the molecule is the essential region for ion channel formation (Mathew and Balatum 1983), the loss of part of the helix in tolaasin-144 would account for the loss of activity in planar lipid bilayers. The fact that this does not correlate with the loss of ability to cause blotch disease symptoms on mushroom caps implies that the mode of action of tolaasin-144 on the intact cap is other than by the unaided formation of ion channels. It is possible that the activity of tolaasin may be facilitated or altered by the presence of an unidentified factor in the wall or membrane of the outer layer(s) of the mushroom cap, although this factor cannot be a requirement for ion channel formation.

ACKNOWLEDGMENTS

We are grateful to J. C. Nutkins, R. J. Mortishire-Smith, and D. H. Williams for helpful discussions. The work was performed under provisions of licence number PHF 1 74A/91 (28) issued by the Ministry of Agriculture, Fisheries and Food under the Plant Health (Great Britain) Order 1987. We thank the Gatsby Charitable Foundation for a Research Studentship (C.L.B.) and the AFRC for a project grant (PG8/509). M. T. is grateful for the Glaxo Junior Research Fellowship at Churchill College, Cambridge, and for grants to Professor E. MacRobbie from SERC, Nuffield Foundation and Royal Society. This work was conducted within the SERC Cambridge Centre for Molecular Recognition.

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